Video Article

In Vivo Nanovector Delivery of a Heart-specific MicroRNA-sponge

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Abstract

MicroRNA (miRNA) is small non-coding RNA which inhibits post-transcriptional messenger RNA (mRNA) expression. Human diseases, such as cancer and cardiovascular disease, have been shown to activate tissue and/or cell-specific miRNA expression associated with disease progression. The inhibition of miRNA expression offers the potential for a therapeutic intervention. However, traditional approaches to inhibit miRNAs, employing antagomir oligonucleotides, affect specific miRNA functions upon global delivery. Herein, we present a protocol for the *in vivo* cardio-specific inhibition of the miR-181 family in a rat model. A miRNA-sponge construct is designed to include 10 repeated anti-miR-181 binding sequences. The cardio-specific α-MHC promoter is cloned into the pEGFP backbone to drive the cardio-specific miR-181 miRNA-sponge expression. To create a stable cell line expressing the miR-181-sponge, myoblast H9c2 cells are transfected with the α-MHC-EGFP-miR-181-sponge construct and sorted by fluorescence-activated cell sorting (FACs) into GFP positive H9c2 cells which are cultured with neomycin (G418). Following stable growth in neomycin, monoclonal cell populations are established by additional FACs and single cell cloning. The resulting myoblast H9c2-miR-181-sponge-GFP cells exhibit a loss of function of miR-181 family members as assessed through the increased expression of miR-181 target proteins and compared to H9c2 cells expressing a scramble non-functional sponge. In addition, we develop a nanovector for the systemic delivery of the miR-181-sponge construct by complexing positively charged liposomal nanoparticles and negatively charged miR-181-sponge plasmids. *In vivo* imaging of GFP reveals that multiple tail vein injections of a nanovector over a three-week period are able to promote a significant expression of the miR-181-sponge in a cardio-specific manner. Importantly, a loss of miR-181 function is observed in the heart tissue but not in the kidney or the liver. The miRNA-sponge is a powerful method to inhibit tissue-specific miRNA expression. Driving the miRNA-sponge expression from a tissue-specific promoter provides specificity for the miRNA inhibition, which can be confined to a targeted organ or tissue. Furthermore, combining nanovector and miRNA-sponge technologies permits an effective delivery and tissue-specific miRNA inhibition *in vivo*.

Video Link

The video component of this article can be found at https://www.jove.com/video/57845/

Introduction

Over the last two decades, there have been numerous studies that have pointed to the significant role of miRNAs in human disease. Findings from a large body of literature demonstrate the undeniable importance of miRNAs in the pathophysiology of diseases, such as cancer[1] and cardiovascular disease[2,3,4,5]. For example, miR-21 is upregulated in many cancers, resulting in an increased cell cycle and cell proliferation[6]. In hepatitis C infections, miR-122 plays an important role in the replication of the virus[7], and it has been shown that the inhibition of miR-122 decreases the viral load[8]. In cardiac hypertrophy, miR-212/132 is upregulated in the heart and is involved in the pathological phenotype[9]. The obvious importance of the downregulation or functional inhibition of an upregulated miRNA suggests opportunities for therapeutically exploiting the miRNA biology in almost all diseases.

The four miR-181 family members, miR-181a/b/c/d, are found in three genomic locations in the human genome. The intronic region of a non-coding RNA host gene (MIR181A1-HG) encodes the cluster of miR-181-a/b-1. The intronic region of the NR6A1 gene encodes the miR-181-a/b-2. The miR-181-c/d cluster is located in an uncharacterized transcript on chromosome 19. All the miR-181 family members share the same "seed" sequence and all four miR-181 family members can potentially regulate the same mRNA targets.

We[3,4] and others[10] have highlighted the importance of miR-181 family members during the end-stage heart failure. We have also recognized that a miR-181c upregulation occurs under pathological conditions associated with an increased risk of heart disease, such as type II diabetes, obesity, and aging[3,4,5]. It has been postulated that the overexpression of miR-181c causes oxidative stress which leads to a cardiac-dysfunction[4].

Several groups have suggested that miRNA exist in mitochondria[11,12,13,14], but we were the first to demonstrate that miR-181c is derived from the nuclear genome, processed, and subsequently translocated to the mitochondria in the RISC[3]. Furthermore, we have detected a low expression

of miR-181a and miR-181b in the mitochondrial compartment of the heart[5]. Importantly, we have found that miR-181c represses the mt-COX1 mRNA expression, thereby demonstrating that miRNAs participate in the mitochondrial gene regulation and alter mitochondrial function[3,4].

This article discusses the methodology required to design a miRNA-sponge to knock down the entire miR-181 family in cardiomyocytes. Moreover, we outline a protocol for the *in vivo* application of the miR-181-sponge.

Protocol

All experimental procedures were approved by the Institutional Animal Care and Use Committee of Johns Hopkins University.

1. Sponge Design

1. **MicroRNA binding 3'UTR**
   Note: A miRNA functions through specific base pairing interactions with partially complementary sites in the 3' untranslated region (UTR) of its target mRNAs (for a comprehensive review, see Bartel)[15].
   1. Design the inhibitors of miRNA expression as oligonucleotides that contain significant complementarity to the miRNA sequence. Sequester a miRNA in an oligonucleotide complex through extensive base pairing to block the miRNA function.

2. **Design of a miRNA sponge**
   1. Design tandemly arrayed partially complementary miRNA sequences to include a bulge at the position normally cleaved by Argonaute 2. The bulge is positioned at nucleotides 9 - 12 of the mature miRNA sequence to prevent any RNA interference type cleavage and degradation of the sponge.
   2. If desired, design the decoy target sequence to bind to all miR-181 family members. Upon an examination and comparison of the miR-181 family miRNA sequences, find a common sequence at the 5'-side of the bulge containing 8 consecutive nucleotides, or find a sequence common to all 4 miR-181 family members on the 3'-side of the bulge of an additional 8 consecutive nucleotides. These 2 sequences represent the left and right half binding sites, respectively.
      Note: The decoy sequence is a tandem array of the left and right half binding sites separated by a GGA spacer. Link together the 5'-end sequence and the 3'-side sequence (described in step 1.2.2) with a GGA spacer designed to create the bulge when annealed to a miRNA (as described in step 1.2.1). Repeat this 1 miRNA binding site 10x in the final miR-sponge construct.

3. **Other miRNA sponge considerations**
   Note: In order to clone the sponge sequence into the recipient expression vector, restriction nuclease recognition sites must be included at each end of the sequence. An *in silico* digestion analysis should be used to confirm the non-specific cleavage of the sponge sequence by the chosen restriction enzymes and other restriction enzymes used in downstream cloning applications.
   1. Add a double stop codon (TAA TAA) at the 5'-end of the synthetic 3'UTR, such that the miR-181-sponge sequence is not part of the coding sequence for an enhanced green fluorescent protein (EGFP).

4. **Synthesis of the sponge DNA for cloning**
   1. Use a DNA synthesizer or employ a commercially available source to synthesize the final miRNA sponge sequence with restriction nuclease recognition sites in place for cloning. The sponge is shipped on a plasmid which can contain selectable markers for an amplification in bacteria. Further, the sponge can be amplified by a polymerase chain reaction (PCR) for cloning or simply dropped out the donor vector using the restriction sites described in step 1.3 (also, see step 3.1).

2. Cloning the Recipient Vector to Include a Tissue-specific Promoter

Note: Use the pEGFP-C1 vector as the recipient vector for the expression cassette of the miRNA sponge. The pEGFP-C1 vector contains a reading frame for an EGFP driven by a cytomegalovirus (CMV) promoter. The CMV promoter is constitutively active in mammalian cells. If a tissue-specific promoter is required, the CMV promoter can be removed by digestion of AseI and NheI enzymes (see step 2.1). The plasmid contains an extensive multiple cloning site (MCS) as well as kanamycin (Kan) and neomycin (G418) markers for a selection in bacteria and a stable expression in mammalian cells, respectively.

1. **Removing the CMV promoter from pEGFP-C1**
   1. Make 50 µL of digestion reaction containing 1x commercially available digest buffer suggested by the manufacture of the restriction enzymes used, 5 µg of plasmid DNA (in water), and 5 µL each of AseI and NheI enzymes. Add all components to a microcentrifuge tube and gently mix them by flicking it. Spin the tube for 10 s in a microcentrifuge to collect the reaction at the bottom. Incubate the reaction in a 37 °C water bath for 15 min.
   2. Purify the digested linearized plasmid. Add 5x the reaction volume of column binding buffer (a proprietary mix of the appropriate binding buffer for the manufacture of the DNA purification columns used) and purify the reaction with a DNA purification column as described by the manufacturer. Elute with 25 µL of elution buffer (water) added carefully to the center of the column.
   3. Gel purify the digested plasmid on 0.5% agarose gel as follows: add 5 µL of loading buffer (50% glycerol-3x loading dye) to the eluted plasmid. Load the entire sample into 1 well on 0.5% agarose gel. Include a separate lane with 500 ng of uncut vector. Run it for 30 - 40 min until an adequate separation of cut and uncut plasmids is achieved.
   4. Purify the linearized plasmid as follows: visualize the DNA in the agarose gel on a UV light box. With a clean razor blade, carefully excise the band corresponding to the linear plasmid.
      Note: The linear plasmid should run lower than the uncut plasmid and will appear as a single band at approximately 4.2 kb. The excised CMV promoter will appear as a light band at approximately 500 nucleotides (nt).
   5. Extract the DNA from the gel slice using a commercially available kit and elute the DNA from the column with 25 µL of water.

2. **Cloning a heart-specific promoter into pEGFP-C1**
   Note: The alpha-myosin heavy chain (α-MHC) promoter has been previously characterized and demonstrated to direct robust levels of cardiac-restricted expression as described in the following Molkentin, Jobe, and Markham[16]. The next sections describe how to amplify the promoter of the vector for cloning.
   1. Clone the α-MHC promoter between elements +420 to -2934 relative to the transcription start site into the p40 plasmid[16] by first designing PCR primers that flank this region and then the plasmid as a template for PCR. Amplify the promoter region using PCR primers previously described[5]. Forward and reverse PCR primers contain sequences of overlap to the digested ends of the pEGFP-C1 (step 2.1) to allow In-Fusion directed cloning. Consult the In-Fusion manual for a comprehensive explanation of the primer design for In-Fusion cloning. Primer sequences are found in **Table 1**.
   2. Prepare a 50 µL PCR reaction, which contains 1 µM of each primer and 10 ng of template DNA. Add an appropriate amount of PCR enzyme and dNTPs depending on the manufacture of the PCR reagents chosen. Perform PCR on a standard DNA cycling block. Perform a 3 min 98 °C denaturing step, followed by 35 cycles of denaturing, annealing, and extension for 5, 30, and 120 s each, respectively. Include a final extension of 10 min at 72 °C.
   3. Clean up the PCR and gel extraction. Upon completion of the PCR cycle, add 5x the reaction volume of the column binding buffer to the PCR reaction and purify it with a DNA purification column as described by the manufacturer. Elute the mixture with 25 µL of elution buffer (water) added carefully to the center of the column.
      1. Add 5 µL of loading buffer [50% glycerol (3x) loading dye] to eluted plasmid. Load the entire sample into 1 well on 1% agarose gel. Run it for 30 - 40 min; visualize and excise the PCR product as described above (step 2.1.5).
   4. Ligate the α-MHC promoter with the pEGFP-C1 as follows: add the 10 µL ligation reaction containing 1x commercially available ligation buffer, 2 µL of purified PCR, and 1 µL of linearized vector. Perform the ligation at 50 °C for 15 min and then cool it on ice.
   5. Perform a bacterial transformation as follows: transfect 50 µL of Competent Cells with 2.5 µL of In-Fusion ligation mix by adding the components. Rest the Eppendorf tube on ice for 20 min, put it in a 42 °C water bath for 40 s, and then place the tube on ice for 2 min.
      1. Following the transformation, add 350 µL of SOC media and grow cells at 37 °C for 45 min. Plate 200 µL of outgrowth solution on LB plates with Kan. Perform a colony PCR to identify Kan resistant cells which contain the α-MHC promoter ligation.
         Note: Screening primers were designed to PCR across the ligation junction.
   6. Expand the PCR positive clones in LB-Kan broth and in plasmids purified using standard mini-prep plasmid purification protocols as described by the manufacturer.

3. Cloning the Sponge

1. **Digest the recipient vector**
   1. Make the α-MHC-pEGFP plasmid linear for cloning with EcoRI and KpnI, and purify the gel as described above (step 2.1).

2. **PCR amplify and digest the sponge DNA**
   1. Use the miR-181-sponge and corresponding 284-nt-long scramble shipping vectors as the template for PCR. Amplify the sponge region using previously described PCR primers[5]. Note that forward and reverse PCR primers contain restriction enzyme sequences to permit ligation into α-MHC-pEGFP-C1. Perform PCR as described above (step 2.2.2). Purify the PCR products prior to digestion as described (step 2.2.3) and eluted them with water for digestion. For primer sequences, see **Table 1.**
   2. Digest the sponge DNA for ligation. A 50 µL digestion reaction contains 1x digest buffer, the gel-purified PCR reaction from step 3.2.1, and 5 µL of both EcoRI and KpnI enzymes. Add all components to a microcentrifuge tube and gently mix them by flicking it. Spin the tube for 10 s in a microcentrifuge to collect the reaction at the bottom. Incubate the reaction in a 37 °C water bath for 15 min.
   3. Purify the digested PCR sponge using a PCR clean-up column as described previously (step 2.2.1).

3. **Ligate the miR-181-sponge into α-MHC-pEGFP-C1 vector**
   1. Set up a 21 µL ligation reaction which contains 1x ligation buffer, 3 µL of digested and purified miR-181-sponge PCR, 1 µL of linearized α-MHC-pEGFP-C1 vector, 1x DNA buffer, and 1 µL of DNA ligase. Perform the ligation at room temperature for 5 min and then place it on ice.
   2. Transform 50 µL of XL1-blue competent cells with 2 µL of ligation mix. Use the transformation and plating protocols as described above (step 2.2.5). Perform a colony PCR to identify Kan resistant bacteria which contain the correct α-MHC-pEGFP-miR-181-sponge sequence. Design screening primers to the PCR across the ligation junction. See **Table 1** for primer sequences.
   3. Amplify and sequence the vector. Expand the PCR positive clones in LB-Kan broth and in plasmids purified using standard mini-prep plasmid purification protocols. Perform a DNA sequencing to verify the plasmid expressing the desired DNA sequences.

4. Generation of Stable H9c2 miR-181-sponge Expressing Cells

1. **Growth and maintenance of H9c2 cells**
   1. Culture the H9c2 cells in Dulbecco's Modified Eagle's Medium (DMEM) containing fetal bovine serum (FBS) to a final concentration of 10%. Sub-culture the cells using standard protocols and grow them at 37 °C in 5% carbon dioxide.

2. **Transfection and selection of H9c2 α-MHC-pEGFP-C1-miR-181-sponge expressing cells**
   1. Perform an electroporation of H9c2 cells for the best results. Transfect rat myoblasts (H9c2) with either a scramble sponge (a 284-nt-long scramble sequence which replaces the miR-181-sponge-sequence) or the α-MHC-pEGFP-miR-181-sponge construct with an electroporator.
      1. Briefly, transfect $4x10^{-5}$ cells with 2 µg of plasmid DNA (in 5 µL of H_2O) and 100 µL of a solution compatible with the electroporation device. Use an electroporation program of DS-120 to transfect the H9c2 cells.

2. Incubate the transfected cells in the cuvette for 10 min at room temperature. Add 500 µL of DMEM directly to the cuvette. Gently transfer the total cuvette content to a well of a 6-well plate.

2. Select for GFP positive cells after 48 h post-transfection by FACs. Plate only the GFP-expressed cells into a 6-well plate with an addition of neomycin (G418) (400ng/mL) to the complete growth media.
   Note: Prior to the G418 selection, a kill curve should be established to determine the amount of neomycin required for the selection. For H9c2 cells, 400 ng/mL of G418 resulted in an approximately 80% death rate of non-plasmid transfected H9c2 cells after 24 h and was considered to be the desired working concentration of G418 for these cells. The cell line was considered stable after growth was maintained in G418 for 16 days.
3. Perform the FACs sorting of the G418 stable cells using the expression of GFP as a marker for a sponge positive selection. Seed 5 - 10 cells in each well of a 96-well plate by flow-sorting. Expand the monoclonal cells from the 96-well plate to 24-well plates over several passages. Use freezer stocks of cells from passage 3 (P3) cells as the starting point for subsequent experiments.
4. Perform all cell-line-based experiments with low-passage cells between P5 and P10.

5. Synthesis and Purification of Sponge-nanoparticles (miR-181-sponge Nanoparticles)

1. Prepare the liposomal nanoparticles by dissolving cationic amphiphile (DOTAP) and co-lipids, cholesterol and DSPE-PEG-OMe, in a 5:5:0.1 mM ratio, respectively, in a mixture of chloroform and methanol in a glass vial.
2. Remove the organic solvent at 44 - 45 °C, under a vacuum by using a rotary evaporator, followed by a gentle flow of moisture-free nitrogen. Keep the remaining dried film of lipid under a high vacuum for 8 h.
3. Prepare a mixture by adding 5% glucose to the vacuum-dried lipid film. Leave it overnight to allow this mixture to hydrate the film. Vortex the vial for 2 to 3 min at room temperature in order to produce multilamellar vesicles with an occasional shaking in a 45° C water bath.
   1. Sonicate the multilamellar vesicles to prepare small unilamellar vesicles in an ice bath for 3 - 4 min until clarity can be observed, at a 100% duty cycle and 25 W output power.

4. Mix either a scramble sequence or a miR-181-sponge sequence cloned into the pEGFP(α-MHC) vectors and liposome on a 1:3 charge ratio basis to form the nanovector[4].
   Note: An electrostatic complex of positively-charged liposomal nanoparticles and negatively-charged plasmid DNA is known as a nanovector.

6. Systemic Delivery of Sponge-nanoparticles and Validation of *In Vivo* Effects

1. **Systemic delivery of sponge-nanoparticles in rats**
   1. Use Sprague-Dawley (SD) rats. Inject the rats intravenously with the miR-181-sponge nanovector or scramble nanovector through the tail vein. Use SD male rats, which are about 200 g in body weight.
   2. Perform the tail vein injection using a dose of 4 mg of nanovector/kg body weight and repeat this using a regimen of 6 tail vein injections over 2 weeks. Following the repeated nanovector delivery, allow a 1-week period (days 15 - 21) for the expression of the vector *in vivo*.
   3. Confirm the optimization by imaging the GFP signal before, during, and after the nanovector delivery. Analyze the GFP signal by tomographic imaging software, which generates semi-quantitative data.

2. **Validation of *in vivo* miR-181 inhibition by the miR-181-sponge**
   1. Perform western blot in order to demonstrate the expression of a functional miR-181-sponge in the heart tissue.
      Note: For this study, mt-COX1 expression was examined.

3. **Functional consequences of *in vivo* miR-181-sponge expression in the heart**
   1. Perform a two-dimensional, M-mode, and Doppler echocardiography during and after the nanovector delivery to analyze the cardiac function and morphological changes. For a better scanning capacity in the rodent hearts, use an ultrasound system equipped with a 15 MHz linear array transducer.
   2. Anesthesia may influence the cardiac contractility; therefore, perform the delivery of the miR-181-sponge nanovector or scramble nanovector to animals that are conscious and without any anesthetic reagent during the echocardiography process. Please see Das, *et al.*[4] for further clarification.

Representative Results

In the stably transfected pEGFP-miR-181-sponge-expressing H9c2 cells (from step 4.2), the expression of the entire miR-181 family (miR-181a, miR-181b, miR-181c, and miR-181d) was moderately decreased relative to pEGFP-scrambled-expressing H9c2 cells. MiR-181-sponge serves as a competitive inhibitor of the entire miR-181 family, so we anticipated that the expression of the miR-181c mitochondrial target gene, mt-COX1, would increase. Western blot data suggest that the mt-COX1 expression was increased in the pEGFP-miR-181-sponge-expressing H9c2 cells compared to the pEGFP-scramble-expressed H9c2 cells. Additionally, no expression changes for either mt-COX2 or mt-COX3 were detected *via* immunoblot in the pEGFP-miR-181-sponge-expressing H9c2 cells. We used α-tubulin as the normalization control for the immunoblotting analysis. GFP signals were mainly observed in the liver and kidney up to 2 weeks post-systemic delivery of the miR-sponge nanovector (**Figure 1A**). However, the GFP expression was significantly higher in the heart tissue after 3 weeks of miR-181-sponge nanovector delivery (**Figure 1A** and **1B**). Of note, there was a GFP signal detected in the liver tissue in some animals 3 weeks after the systemic delivery; however, there were no functional effects of the miR-181-sponge in the liver at that time point.

The western blot showed a significant increase in the mt-COX1 expression in the miR-181-sponge nanovector-injected rats compared to the scramble nanovector groups (**Figure 2**). Higher mt-COX1 suggests there is a lower miR-181c expression in the heart after 3 weeks of the miR-181-sponge nanovector delivery, as mt-COX1 is the direct target for miR-181c. We used α-tubulin as the normalization control for the immunoblots.

Echocardiography showed no change in the cardiac function of the miR-181-sponge nanovector-injected rats before, during, and at the end of the treatment regimen, compared to the scramble nanovector group of animals.

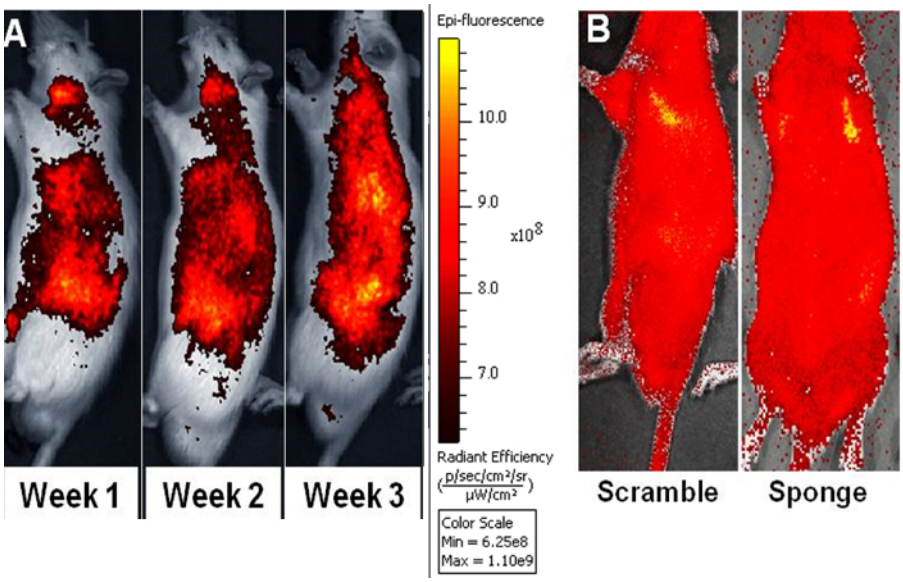

Figure 1. *In vivo* analysis of nanovector delivery of the miR-181-Sponge. (**A**) This panel shows the optimized 3-week treatment protocols with 6 intravenous injections through the tail vein. The yellow colorization in the epifluorescence image demonstrates the expression of the pEGFP vector. The maximum intensity of the yellow colorization is observed at the week 3 time point. (**B**) The α-MHC promoter sequence in the pEGFP vector selectively expresses either the miR-181-sponge or the scrambled sequence in the heart at day 21. Please click here to view a larger version of this figure.

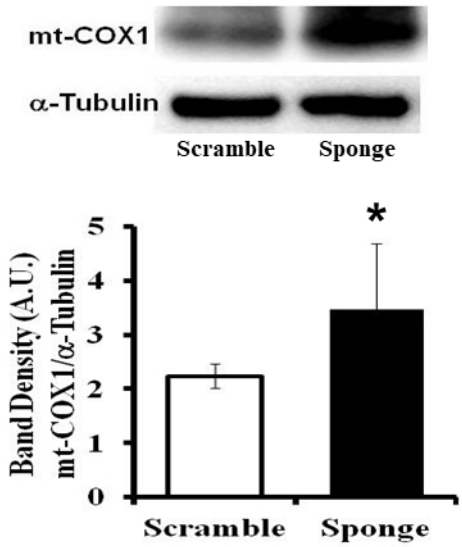

Figure 2. Validation of the effect of miR-181-sponge nanovector on miR-181c expression. This is the western blot analysis of the mt-COX1 expression in the heart lysates obtained from pEGFP-scramble and pEGFP-miR-181-sponge nanovector-injected rats. Whole-heart homogenates were probed with the indicated antibodies. We used α-tubulin as a loading control. The band-densitometry is shown in the bar graph below. Student's *t*-test was performed, and the standard error was plotted in the bar graph as error bars. * p <0.05 *vs.* pEGFP-scramble group. n = 4.

Primer Name	Sequence	TM	Notes	Use	Target
SAM3-1F	ATGCATTAGTTATTAATGCTT GACACACTTGACAATTTCT	58.4	Rat MHC promoter to clone into EGFP	PCR	MHC promoter
SAM3-2R	GACCGGTAGCGCTAGCTG ACTCACTGGGAGATTGCTT	59.8	Rat MHC promoter to clone into EGFP	PCR	MHC promoter
SAM3-3F	CGAACGACCTACACCGAACT	64	Screen EGFP-F	Colony PCR	Positive promoter clones
SAM3-4R	CGCTAGTCCTTGACCCTCTG	63.9	Screen MHC-R	Colony PCR	Positive promoter clones
SAM5-1F	CCTGTCTCCAACACACAAGC	59.3	Sequence MHC promoter	Sequencing	MHC promoter
SAM5-2R	CAGACTGCAGGGCTGGTT	60	Sequence MHC promoter	Sequencing	MHC promoter

Table 1. Primer sequences.

Discussion

This article described the design and synthesis of a miRNA-sponge and demonstrated how the tissue-specific expression of the sponge is a powerful tool to inhibit tissue-specific miRNA family expression.

We have demonstrated that a miR-181 family targeting sponge can be cloned into an expression plasmid with a cardiac-specific promoter. The plasmid can be efficiently packaged into a nanovector particle for delivery both *in vitro* and *in vivo* using electroporation or a tail vein injection, respectively (**Figure 1**). The miR-181 sponge can inhibit a cardiac-specific expression of the miR-181 family and can affect the expression of miR-181 target genes (**Figure 2**). The GFP in the plasmid is an added advantage, which can visualize the delivery and the expression profile of the nanovector without sacrificing the animals.

Briefly, a miRNA-sponge consists of a series of miRNA antisense sequences placed after a reporter gene that acts as a decoy miRNA target mRNA. Naturally occurring miRNA-sponges have been found to be endogenously expressed in plants and animal cells as long non-coding RNAs (lncRNAs)[17]. In the present study, we have utilized a sponge approach to inhibit the entire miR-181 family in the heart. Although the miR-181-sponge approach is a physiologically relevant method to downregulate the miR-181 family in cultured cells, an *in vivo* application of miR-181-sponges can be detrimental. For example, several studies have demonstrated a protective role of miR-181a and miR-181b in different cell/tissue types[18,19,20]. Therefore, the miR-181-sponge expression should be targeted specifically to heart tissue.

Small oligonucleotides have been demonstrated to block the miRNA function through annealing to the mature miRNA guide strand and prevent a proper loading into the RNA-induced silencing complex (RISC). A problem associated with this approach is that oligonucleotides have to be delivered in a saturating dose sufficient to block cellular pools of mature miRNAs. Oligonucleotides also suffer from challenges involving transport across cellular membranes and a general stability of the molecule. Ingeniously, it has been shown that an exogenously supplied miRNA target can act as a decoy for its cognate miRNA[21]. By virtue of multiple binding sites tethered together into a pseudo 3'UTR construct, the decoy target, or miRNA-sponge, is able to stably interact with its target miRNAs and sequester the miRNA into microribonucleoprotein complexes (miRNPs), thus effectively preventing the miRNA function. The so-called "miRNA-sponge" is an effective anti-sense technology, which can efficiently downregulate miRNA both *in vitro* and *in vivo*. Therefore, the application of a miRNA-sponge can be used to study miRNA loss-of-function phenotypes.

The notion that RNA interference (RNAi) could lead to a new class of therapeutics caught the attention of many investigators soon after its discovery. The field of applied RNAi therapeutics has moved very quickly from lab to bedside. Presently, miRNA therapeutics is one of the fast-growing therapeutic interventions in oncology and is currently in phase 1 clinical trials. Antisense oligonucleotides, or antagomirs, are one of the most widely used miRNA inhibitory approaches. Ma *et al.*[22] used miR-10b antagomir, both *in vitro* and *in vivo*, to silence upregulated miR-10b in a solid tumor.

In vivo, the efficient silencing of upregulated miRNAs requires a chemical modification of the antagomirs to improve the binding affinity, biostability, and pharmacokinetic properties. To increase the duplex melting temperature (T_m) and improve the nuclease resistance of antagomirs, chemical modifications can be performed, such as 2′-O-methyl (2′-O-Me), 2′-O-methoxyethyl (2′-MOE) 2′-fluoro, and the bicyclic-locked nucleic acid (LNA)[23,24,25,26,27,28,29,30,31]. For a better efficiency of the *in vivo* delivery, the antagomir can be modified by the phosphorothioate (PS) linkages, which have increased the nuclease resistance[28]. Additionally, PS backbone modifications also enhance the binding to plasma proteins reducing urinary excretion. Thus, PS-modified antagomirs show a significant improvement of pharmacokinetic properties, facilitating their systemic delivery[32]. It has also been shown that using peptide nucleic acid (PNA) or morpholino oligomers, designed to target a specific miRNA, can be used to study miRNA loss-of-function phenotypes[33,34,35,36,37,38,39]. Polylysine-conjugated and nanoparticle-complexed PNA antagomirs efficiently inhibit the miRNA function both *in vitro* and *in vivo*[36,37,38,39]. Despite recent advances, an effective and tissue-specific delivery of miRNA-derivatives remains a challenge. These include the potential for off-target effects, triggering innate immune responses and, most importantly, obtaining a specific delivery into the cells of targeted organs/tissues.

Additionally, miRNA expression levels vary greatly depending on the cell and tissue type[40]. Furthermore, the miRNA expression can be increased or decreased in disease. The consequence of an altered miRNA expression and the effect on cellular and tissue function with sponge-inhibited miRNA expression needs to be validated and determined empirically. Extensive preclinical studies in animal disease models are needed to determine the optimal level of the inhibition for a given miRNA target.

Interestingly, miR-181c miRNA exhibits subcellular compartmentalization[3,4,5]. Specifically, as expected, the miR-181s are encoded in the nucleus and transcribed as long-pri-miRNA transcripts which are processed in the cytoplasm by the dicing machinery and incorporated into RISC. However, unlike canonical miRNAs that function in the cytoplasm, the mature form of miR-181c translocates into the mitochondria and functions in the regulation of mitochondrial-specific genes[3]. We have demonstrated that the miR-181-sponge can bind the mature form of miR-181c in the cytoplasmic fraction, thus preventing the translocation to the mitochondria. Consequently, an upregulation of mt-COX1 can be observed in the mitochondria under the conditions of a downregulation of the miR-181c expression.

We have reported the methodology required to design a miRNA-sponge to knock down the expression of any miRNA and outlined a protocol for the *in vivo* application of the miRNA-sponge. Tissue-specific miRNA inhibition is currently an under-developed technique in the miRNA field. The importance of downregulation or functional inhibition of upregulated miRNAs in human disease suggests that it may be possible to exploit miRNA biology for therapeutic intervention.

This study highlighted a strategy for lowering a miRNA, which can be successfully employed in a tissue-specific manner *in vivo*. The miRNA-sponges utilize the antisense sequence of the miRNA "seed" sequence. Therefore, miRNA-sponges can downregulate all of the miRNAs that share the same "seed" sequence, *viz.*, the entire miRNA family. In this study, we have observed a significant downregulation of the entire miR-181 family (miR-181a, miR-181b, miR-181c, and miR-181d) with the expression of the miR-181-sponge, both *in vitro* and *in vivo*. If one family member confers protection while another family member has detrimental effects within the same organ, using the miRNA-sponge technology would not be the ideal approach.

Disclosures

The authors have nothing to disclose.

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