Protocol

Intracranial delivery of antisense locked nucleic acids for silencing microRNAs in the mouse brain

Antisense locked nucleic acid (LNA) technology has been widely used for silencing microRNAs with enhanced specificity and efficiency. In this protocol, we first describe the procedure for targeted intracranial delivery of LNAs to silence microRNAs specifically in the mouse brain. We then detail the steps to isolate RNA and protein from mouse brain, followed by using RT-PCR and Western blotting to confirm microRNA silencing. This noninvasive approach can only be applied to mouse brain to specifically target silencing of microRNAs.

Publisher’s note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.
Intracranial delivery of antisense locked nucleic acids for silencing microRNAs in the mouse brain

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SUMMARY

Antisense locked nucleic acid (LNA) technology has been widely used for silencing microRNAs with enhanced specificity and efficiency. In this protocol, we first describe the procedure for targeted intracranial delivery of LNAs to silence microRNAs specifically in the mouse brain. We then detail the steps to isolate RNA and protein from mouse brain, followed by using RT-PCR and Western blotting to confirm microRNA silencing. This noninvasive approach can only be applied to mouse brain to specifically target silencing of microRNAs.

For complete details on the use and execution of this protocol, please refer to Sharma et al. (2021).\textsuperscript{1}

BEFORE YOU BEGIN

Introduction

MicroRNAs are small noncoding RNAs that can regulate many cellular functions.\textsuperscript{2} Exogenous manipulation of these cellular microRNA levels has been used as a tool to understand the role of various microRNAs in regulating cellular signaling mechanisms.\textsuperscript{3} MicroRNA inhibitors (or Anti-miRs) are antisense oligonucleotides which bind to complementary microRNAs and block their action. In recent years, different chemical modifications of these RNAs (e.g., cholesterol conjugation, introduction of Locked nucleic acid chain, 2'-O-methyl nucleotides) have been designed for increasing their stability in vivo.\textsuperscript{4} To study the roles of specific microRNAs in different tissues of mice, miRNAs or Anti-miRs can be delivered to them by various routes e.g., intraperitoneal, intravenous, intranasal.\textsuperscript{5} Genetic knockout studies and use of antisense oligonucleotides are used for studying loss of function in vivo. Chemically modified antisense oligonucleotides targeting specific microRNA are widely utilized to sequester microRNAs causing de-repression of target mRNAs.\textsuperscript{4} In vivo use of antisense nucleotides needs to be optimized for their enhanced binding affinity and nuclease resistance in animal tissues. In our study\textsuperscript{1} comprising the use of antimiR-24 for silencing miR-24 in brain, customized antisense nucleotides (LNA) were injected into the cranium of mice. LNAs are bicyclic RNA analogs in which the furanose ring of the ribose sugar-phosphate backbone is chemically locked due to the introduction of a 2'-O,4'-C methylene bridge mimicking N-type (C3'-endo) conformation.\textsuperscript{6,7} This modification enhances nuclease resistance and binding affinity of antisense nucleotides to the complementary microRNA due to higher duplex melting temperature (Tm value). Apart from using LNA nucleotides, another common modification is the modification of phosphodiester linkages into phosphorothioate (PS) backbone in which one of the non-bridging oxygen group is replaced by sulfur atom in the phosphate group.\textsuperscript{8} The protocol below describes an easy noninvasive method for intracranial delivery of antisense nucleotides targeting miRNA-24 for achieving brain specific silencing of miRNA-24. This protocol will help the readers further to follow our study better and perform similar experiments in the future with precision.
Ethical statement
All animal experiments were performed in compliance with protocols approved by the Cleveland Clinic Institutional Animal Care and Use Committee.

Note: Ethical permission from Institutional regulatory committee is mandatory before conducting this experiment.

Preparation of LNA solution

- Timing: 10 min

1. Dissolve 15 pmol LNA oligonucleotide in 300 µL nuclease-free water so that the final concentration is 1 pmol / 20 µL. Customized oligonucleotides are sold by Qiagen in lyophilized form which are dissolved and stored at -20 degree.

Note: Store small aliquots at -20 degree to avoid freeze thaw cycles.

2. Procure thin needles (26 G 5/8 gauge) and 0.5 mL sterile syringes.

Preparation of anesthesia

- Timing: 5 min

3. Prepare a mixture of ketamine and xylazine in sterile PBS accordingly (final volume 2 mL).

| Reagent          | Stock conc | Working conc | Volume to be added |
|------------------|------------|--------------|--------------------|
| Ketamine         | 100 mg/mL  | 25 mg/mL     | 500 µL             |
| Xylazine         | 100 mg/mL  | 2.5 mg/mL    | 50 µL              |
| Sterile PBS      | 1,450 µL   |              |                    |

Note: Make fresh anesthesia mixture. The anesthesia mixture can be stored at 4°C, for maximum 7–8 days.

KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Antibodies          |        |            |
| Anti-STING antibody | Cell signaling technology | 13647S (1:1000 dilution) |
| Anti-actin antibody | Cell Signaling Technology | 3700 (1:10000 dilution) |
| Anti-Myc protein antibody | Santa Cruz Biotechnology | Sc-40 (1:1000 dilution) |
| Chemicals, peptides, and recombinant proteins | | |
| 4% paraformaldehyde in PBS | Thermo Fisher Scientific | J61899 AP |
| Ketamine hydrochloride (ketaset) | Zoetis Inc | 100 mg/mL stock |
| Xylazine | Patterson Veterinary | 100 mg/mL stock |
| Sterile PBS (endotoxin free) | MilliporeSigma | 806552-1L |
| Critical commercial assays | | |
| Taqman Universal RT-PCR master mix | Thermo Fisher Scientific | cat no-4304437 |
| Taqman microRNA assay specific to miR-24 | Thermo Fisher Scientific | catalog no - A25576, assay i.d 481011 |
| Taqman microRNA assay specific to miR-23b | Thermo Fisher Scientific | Catalog-4427975, assay i.d- 000400 |
| Taqman microRNA reverse transcriptase kit | Thermo Fisher Scientific | cat no - 4366596 |
| RNase control assay | Thermo Fisher Scientific | 4427975, assay i.d 001006 |
| miRNA PARIS kit for RNA/ protein isolation | Thermo Fisher Scientific | AM 1556 |

(Continued on next page)
Anesthesia

**Timing:** 5 min

This section describes the procedure to anesthetize the mice.

1. Weigh the mice before giving anesthesia and inject the anesthesia mixture into the peritoneum (I.P) of 6 weeks old C57/Bl6 male mice.
2. Inject 50 μL of anesthesia into mice weighing 17–20 grams.

*Note:* This dose is used for mice ranging 5–6 weeks which weigh (16–20 grams) to temporarily anesthetize the mice. Mice with higher weight or age are not used for I.C injection as their cranium gets harder to inject. Confirm the effect of anesthesia by pinching the tail or foot pad of mice and do not proceed until they are completely unconscious.

Injection of the oligonucleotide into the cranium

**Timing:** 5 min

This section describes injection of oligonucleotide precisely into the cranium of mice and avoid any bleeding from site of injection.
3. Dissolve oligonucleotides and fill the solution into 0.5 mL syringe with thin needle (26 G \(\frac{5}{8}\) gauge).
4. Wipe the head region of mice (no shaving required) with 70% alcohol and hold the head of the mice from the side of its neck with left hand.
5. Very slowly start piercing the needle of syringe into the left side of the cranium at the frontal side of the cortex (left cerebral area as shown in Figure 1).

**Note:** Once the needle tip pierces into the cranium, no resistance to the needle is felt. Do not move the needle further into the brain as it may disrupt brain vessels and may cause bleeding.

6. As soon as the tip of needle penetrates the cranium, do not pierce the needle further deep into the brain and inject 20 \(\mu\)L of antimiR solution into cranium. The position of needle in the cranium is depicted in Figure 1.
7. Slowly take the needle out of the brain and observe the site of injection carefully in order to check bleeding or popping out of the solution. Any leakage or bleeding will be clearly visible at site of injection.

**Note:** The solution should not leak out from the site of injection for efficient delivery of the oligonucleotide to the brain. Figure 1 depicts injection of antimiR efficiently into cranium without causing any bleeding from site of injection.

**Recovery from anesthesia**

© Timing: 20 min

This section describes the recovery of mice after intracranial injection.

8. Keep the mice on a heating pad for some time until they start regaining consciousness.
9. Place the mice back into the cage and monitor them until they start moving.
10. Place a diet gel so that they have easy source of food in the cage.

**Observation of injected mice**

© Timing: 4 days
This section describes observing injected mice for any symptoms and monitoring body weight.

11. Observe the mice daily and monitor its body weight.

**Harvesting the injected mice**

- **Timing:** 30 min

This section describes perfusion of mouse brain with paraformaldehyde and isolation after 4 days post injection weight.

12. After 4 days, inject a terminal dose of anesthesia into the peritoneum of mice and allow the mice to lose consciousness.

**Note:** 80 μL of anesthesia with mixture of ketamine and xylazine used at same final concentration as mentioned above is considered terminal dose for mice weighing 17–20 grams.

13. Place the mice on a surgical pad and make an incision to expose their hearts (on the ventral side of the body).

14. Inject 4% paraformaldehyde solution in PBS into the beating hearts of mice to drain out blood from the blood vessels.

**Note:** Perfusion is done only to remove blood from blood vessels.

15. Then make an incision at the snout area between eyes (dorsal side) and remove the skin to expose the cranium.

16. Cut the cranium by using scissors and isolate the brain. Snap chill brain tissue in liquid nitrogen and store them at -80 degree.

**Confirmation of silencing of miRNA-24 in mouse brain**

- **Timing:** 3–4 h

This section describes isolation of RNA and protein from mouse brain and confirmation of miRNA-24 silencing by RT-PCR and Western blotting.

17. Confirm the silencing of miR-24 in mouse brains by RT-PCR using a miR-24 specific Taqman probe. For extraction of miRNA and protein from same tissue, use the miRVana PARiS kit using manufacturer protocol available online.

https://www.thermofisher.com/documentconnect/documentconnect.html?url=https://assets.thermofisher.com/TFS-Assets%2FLSG%2Fmanuals%2FFfm_1556.pdf.

**Note:** To ensure that the injected oligonucleotide has been well absorbed in the brain tissue, use the right hemisphere for RNA and protein isolation (as the oligonucleotide is injected into the left side) for confirmation of miR-24 silencing.

18. Reverse-transcribe the extracted RNA into cDNA using Taqman reverse transcriptase kit following the manufacturer protocol:

https://genome.med.harvard.edu/documents/qpcr/microRNATaqManAssayProtocol.pdf.

19. Perform RT-PCR using the Taqman probes specific to miR-24 using manufacturer protocol.
20. Silencing of microRNA causes de-repression of the target mRNA. Confirm the silencing of miR-24 by assessing the expression of STING and Myc protein (which are direct targets of miR-24) through Western blotting.

EXPECTED OUTCOMES

Brain tissues are homogenized and miRNA / protein is isolated by using the miRVana PARIS kit (Thermo Fisher Scientific). Successful in vivo delivery of the oligonucleotide blocks miR-24 action and de-repress its target mRNAs expression. In mice injected with a miR-24 targeting oligonucleotide, miR-24 is not detected by RT-PCR analysis of brain RNA (Figure 2A). Also the enhanced expression of STING protein is confirmed by Western blotting (Figure 2B). AntimiR-24 injection into the brain specifically targets miR-24; not other microRNAs. The expression of other non-targeting microRNA (miR-23b) which gets expressed from same gene cluster along with miR-24, is also examined to confirm specific targeting by antimiR-24 (Figure 2C). AntimiR-24 does not affect the expression of miR-23b and specifically silence miR-24 only. As miR-24 also targets expression of Myc protein,10 antimiR-24 inoculation enhances the expression of Myc protein in mouse brain (Figure 3A). As a negative control, a non-targeting LNA oligonucleotide is injected into the mouse brains. These results confirm the specific silencing of miR-24 by antimiR-24 in mouse brain. Because the oligonucleotide is injected to the left side of brains, isolate the RNA and protein from the right side of the brain cortex. This ensures the oligonucleotides are well absorbed throughout the brain and effectively silence miR-24 in the whole brain. Also, RNA and protein is isolated from the same brain tissue.

https://genome.med.harvard.edu/documents/qpcr/microRNATaqManAssayProtocol.pdf.
by using miRVana PARIS Kit) to avoid any discrepancy in results. Injection of oligonucleotides into
the brain does not cause any significant body weight loss (Figure 3C) in mice.

QUANTIFICATION AND STATISTICAL ANALYSIS

Statistical analyses were performed using Graph Pad Prism 5.02 software. The mean ± SEM of all
biological replicates was used to make all graphs. For analyzing body weight changes, percentage
of body weight was plotted using Microsoft excel sheet. ImageJ software was used for quantification
of Western blots.

LIMITATIONS

This protocol is used for localized silencing microRNAs only in mouse brain by intracranial injection.
This I.C injection of antimiR will not silence miRNA in other parts or organs of mice. It does not guar-
antee long term systemic silencing of miRNAs. This protocol cannot be used for intracranial injection
of mice more than 6 weeks of age, as the cranium of mice gets harder with age. Moreover volume
more 20 μL will cause leakage from site of injection.

TROUBLESHOOTING

Problem 1

While injecting into the cranium, excessive penetration of needle into brain may cause bleeding and
oligonucleotides may flow out of the cranium (step 5).

Potential solution

The needle should be used with thickness mentioned above only (26 G 5/8 gauge). Once tip of the needle
enters into the cranium, stop inserting it further into the brain and inject the oligonucleotide very gently.
Problem 2
Mice may appear sick and display neurological symptoms like paralyzed limbs, impeded movements or severe weight loss 1–2 days post injection due to internal bleeding or any other damage to the brain tissue (step 11).

Potential solution
Such mice should not be used for further studies.

Problem 3
The oligonucleotide solution may ooze out from site of injection (step 6).

Potential solution
Do not inject more than 20 µL of solution as more volume will ooze out from the site of injection and cause leakage of the oligonucleotide.

Problem 4
Excessive I.P injection of anesthesia may lead to death of mice (step 2).

Potential solution
Carefully weigh all the mice and inject anesthesia accordingly. For 16–20 gm mice, 50 µL anesthesia should be given (final concentration of ketamine 25 mg/mL and xylazine is 2.5 mg/mL).

Problem 5
Clogging of spin columns while isolating RNA from brain tissue (step 17).

Potential solution
Do not use excess tissue to isolate miRNA / protein as it can clog the columns and reduce the yield. Use 100 gm of brain tissue for miRNA / protein isolation.

RESOURCE AVAILABILITY

Lead contact
Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Ganes C. Sen (seng@ccf.org).

Materials availability
The study did not generate any new unique reagents.

Data and code availability
This study did not generate unique codes. The data generated during this study have been published by the authors.¹

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AUTHOR CONTRIBUTIONS
N.S. performed the experiments, analyzed the data, and wrote parts of the paper, and G.C.S. designed experiments, interpreted the results, acquired funding, and wrote parts of the paper.

DECLARATION OF INTERESTS
The authors declare no competing interests.
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