Protocol to knock in or out target genes in mouse epidydimal white adipose tissue by spot injecting adeno-associated viruses

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Publisher’s note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.
Protocol to knock in or out target genes in mouse epidydimal white adipose tissue by spot injecting adeno-associated viruses

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SUMMARY
Visceral fat obesity is more strongly associated with ectopic fat deposition, lipotoxicity, and metabolic disease compared to generalized obesity. To study the function of visceral fat tissue, we describe steps to knock in or out target genes by spot injecting adeno-associated viruses (AAV) in visceral fat tissue. We provide details on anesthesia, incision, and spot injection into the epidydimal white adipose tissue (eWAT) of live anesthetized mice. Furthermore, we detail an efficient technique for expressing exogenous protein in mouse eWAT. For complete details on the use and execution of this protocol, please refer to Zhao et al. (2022).

BEFORE YOU BEGIN
The specific steps for performing spot injection adeno-associated virus (AAV) in the visceral fat tissue of C57BL/6 mice is described in the protocol below.

Institutional permissions
C57BL/6 male mice (8 weeks old, 21–22 g) were used in this study. All animal experiments were conducted under protocols approved by the Animal Research Committee of the Institute of Laboratory Animals, Institute of Basic Medical Sciences Chinese Academy of Medical Sciences, and School of Basic Medicine Peking Union Medical College (ACUC-A01-2021-017). All mice were housed in the specific pathogen free (SPF) facility and maintained on a 12 h light-dark cycle and a regular unrestricted diet.

AAV packaging
© Timing: 6–8 weeks

Zinarelli et al. showed AAV serotype 9 has the best viral genome distribution and highest protein levels (Zinarelli et al., 2008). Therefore, AAV9 was selected to spot inject in the visceral fat tissue.
1. Select a target gene and construct the coding sequence (CDS) region into the AAV expression vector (the shuttle plasmids).

2. Transfect the constructed shuttle plasmid, AAV9-cap plasmid and pHelper plasmid, into HEK293T cells.

3. Collect and purify AAV9 viral particles 72 h post transfection in accordance with previous study (Wang et al., 2016).

4. Confirm AAV titers by qPCR and adjust to \(10^{10}\) plaque-forming units per mL in PBS containing 4% sucrose.

5. The above experimental procedures were performed in HanBio Co. Ltd.

**Note:** AAV-related experiments require operation in a biosafety cabinet (BL-2 level). All handlers should wear protective equipment including a lab coat and two pairs of gloves. It is crucial that the skin on the hands and arms is not exposed to the virus.

Special care should be taken when handling viruses to prevent spillage. If there is virus contamination in the biosafety cabinet, wipe it off immediately with 70% ethanol and 1% SDS solution.

Soak the pipette tips, centrifuge tubes and culture bottles with 84 disinfection solution and then dispose of them uniformly. If centrifugation is required during the experiment, use well-sealed centrifuge tubes, and if necessary, seal them with parafilm before centrifugation. Virus-related waste should be collected and disposed after autoclaving.

Hands should be washed with soap after the experiment.

If the collected AAV needs to be used within a short period of time, the virus can be stored at 4°C and used it within a week.

**Preparation of surgical instruments**

© **Timing:** 4–6 h

Sterilize surgical instruments by autoclave or steam sterilization.

**Optimize surgeries in the euthanized mice prior to use in vivo**

© **Timing:** 1–2 days
6. Become familiar with the basic surgical instruments (Figure 1) and the surgical procedure to expose and handle eWAT.  
7. Become familiar with suturing procedures and mouse anatomy before performing surgery in vivo.  
8. Select an appropriate anesthetic according to the type of experiment or surgery and the time needed for anesthesia.

**KEY RESOURCES TABLE**

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| **Antibodies** | | |
| anti-β-Actin, dil:1/10000 | Abclonal | Cat#AC026 RRID AB_2768234 |
| anti-STEAP4, dil:1/1000 | Thermo Fisher Scientific | Cat#PA5-106509 RRID: AB_2854178 |
| **Bacterial and virus strains** | | |
| AAV9-CMV-mSteap4-MYC | HanBio Biotechnology (Shanghai) Co., Ltd | N/A |
| AAV9-CMV-ZsGreen | HanBio Biotechnology (Shanghai) Co., Ltd | N/A |
| **Chemicals, peptides, and recombinant proteins** | | |
| Pentobarbital sodium salt | Scientific Research Special | Cat#57-33-0 |
| Ethanol absolute | Sinopharm Chemical Reagent Co. | Cat#64-17-5 |
| 5x SDS-Loading Buffer | Beyotime Biotechnology | Cat#P0015 |
| NaCl | Sinopharm Chemical Reagent Co. | Cat#7647-14-5 |
| DMEM | Thermo Fisher Scientific | Cat#11965-118 |
| Fetal calf serum | Thermo Fisher Scientific | Cat#26050-070 |
| Trypsin | Thermo Fisher Scientific | Cat# LP0042 |
| PBS | Servicebio | Cat#G4202 |
| PageRuler™ Prestained Protein Ladder | Thermo Fisher Scientific | Cat#26616 |
| RIPA buffer | Beyotime Biotechnology | Cat#P0013 |
| Phenylmethanesulfonyl fluoride (PMSF) (100 mM) | Beyotime Biotechnology | Cat#ST506 |
| cComplete Tablets EDTA-free, EASYpack | Roche | Cat#4693132001 |
| 84 disinfection solution | JINGBL | Cat#100038063502 |
| Parafilm | Parafilm | Cat#AM-PM996 |
| Sucrose | Sigma-Aldrich | Cat# V900116 |
| **Experimental models: Cell lines** | | |
| HEK293T | ATCC | Cat#CRL-3216 |
| **Experimental models: Organisms/strains** | | |
| Mouse (Mus musculus): C57BL/6, males | Huafukang Bioscience | Cat#11001A |
| **Software and algorithms** | | |
| BioRender | BioRender | https://biorender.com |
| Viamaker | TikTok | https://lv.ulikecam.com/ |
| **Other** | | |
| 29-gauge insulin syringe | BD | Cat#328421 |
| 15 mL centrifuge tube | Servicebio | Cat#EP-1501-J |
| Fine scissors sharp | Shanghai Medical Equipment (Group) Co. | Cat#Y00030 |
| Ophthalmic forceps (10 cm, curved with hook) | Shanghai Medical Equipment (Group) Co. | Cat#JD1060 |
| Needle holder | Shanghai Medical Equipment (Group) Co. | Cat#J32010 |
| 4-0 Coated VICRYL Plus (Polyglactin 910) Synthetic Absorbable Suture | Ethicon, Inc. | Cat#VCP771D |
| PVDF membranes | Millipore | Cat#IPVH00010 |
| Nature textured latex medical examination gloves | Medicom | Cat#1188A |
MATERIALS AND EQUIPMENT

0.9% NaCl solution

| Reagent         | Final concentration | Amount |
|-----------------|---------------------|--------|
| NaCl powder     | N/A                 | 0.9 g  |
| Pure water      | N/A                 | 10 mL  |
| **Total**       | 0.9%               | 10 mL  |

Pentobarbital sodium solution

| Reagent                        | Final concentration | Amount |
|--------------------------------|---------------------|--------|
| Pentobarbital sodium salt      | N/A                 | 30 mg  |
| Anhydrous ethanol              | N/A                 | 1 mL   |
| 0.9% NaCl solution             | N/A                 | 9 mL   |
| **Total**                      | 3 mg/mL             | 10 mL  |

Note: This solution can be stored away from light at 4°C for one week, and be used in multiple experiments.

STEP-BY-STEP METHOD DETAILS

In this protocol, we will describe how to inject AAV9 in eWAT of anesthetized mice. The method details are outlined in the following protocol and video (Methods video S1).

Anesthetizing mice prior to surgical procedures

◎ Timing: 5 min

1. Randomly divide eight-week-old male C57BL/6 mice into two groups: the experimental group and the control group.
2. Weigh the mouse before injection of anesthetic solution.
3. Intraperitoneally inject 0.1–0.2 mL of anesthetic mixture per 10 g of body weight.
4. Place the mouse back in another clean cage until it is immobilized.

Note: Decreased muscle tone in the mouse, such as pedal withdrawal reflex disappearance, is proved to be completely anesthetized. Anesthetized mice should never be placed in a cage with non-anesthetized mice for prolonged periods as anesthesia leaves them vulnerable to attacks by cage mates.

Note: The safety range of pentobarbital sodium is narrow. The dose of anesthetic must be administered to ensure that the mice remain anesthetized during the surgical procedure and wake up after the surgical procedure, as soon as possible. Take care of the dosage of anesthetics to avoid overdosage. An additional volume might be needed to achieve full anesthetic effect. In addition, the dosage of anesthetic needs to be adjusted according to the strain of the mice. Alternatively, other injectable anesthesia (e.g., the mixture of ketamine and xylazine) and inhalation anesthesia (e.g., isoflurane) can be taken into consideration.

Note: Keep airway patency of the anesthetic mouse, so that there is no excessive secretion that could block the trachea and cause the animal to suffocate.

AAV9 spot injection

◎ Timing: 20–30 min
5. Place the anesthetized mouse in the supine position.
6. Fix the limbs with adhesive tape on a small workbench on a sterile operating table and expose the skin on the abdomen of the mouse adequately.
7. Shorten the fur of mouse for the incision on the abdomen with hair clipper (Figure 2A).
8. Disinfect the mouse skin with 75% alcohol at the site where you wish to make a surgical incision.
9. Make an approximately 0.7 cm in length incision along the ventrimeson in the median abdomen with fine scissors sharp and cut each layer of the abdominal wall, successively (Figure 2B).
10. Locate and fully expose the eWAT by blunt separation with an ophthalmic forceps (10 cm, curved with hook).
   a. Inject the AAV in the left eWAT (relative to midline of mouse abdomen) at 3 points inject 5 μL of the AVV, using a 1 mL sterile BD 29-gauge insulin syringe (Figure 2C).
   b. Repeat for the right eWAT.

**Note:** A 3:1 ratio of anhydrous ethanol to pure water can be mixed to make 75% alcohol.

**Note:** The eWAT is strip shaped. the center of the eWAT is selected as one injection point, and the midpoint between the center point and the two endpoints of the eWAT are selected as the other two injection points.

11. Arrange and reposition the eWAT back into the mouse’s abdominal cavity with an ophthalmic forceps.
12. Simple interrupted suture each layer of the abdominal wall of the mouse intermittently after injection with 4-0 Coated VICRYL Plus (Polyglactin 910) Synthetic Absorbable Suture (Figure 2D).
13. After operation, the mouse is individually placed in a clean cage. Keep the mice warm until they recover from anesthesia.
14. (Optional) Local anesthetics such as lidocaine can be used to relieve local pain and itching.

**Optional:** Identification the effect of spot injection of AAV by Western blotting.
15. Extract protein from fresh adipose tissue samples into RIPA buffer. The protein extraction was performed as previously described (Zhao et al., 2022).
16. Load protein onto a 10% SDS-polyacrylamide gel, and transfer separated proteins to PVDF membranes.
17. Perform Western blot assays using specific antibodies.

Pause point: The experimental mice are housed in the SPF facility and maintained on a 12 h light-dark cycle until the eWAT are collected and the exogenous protein is detected.

EXPECTED OUTCOMES

In our work (Zhao et al., 2022), we used this protocol to produce the overexpression of mouse six-transmembrane epithelial antigen of prostate 4 (STEAP4) protein within the eWAT of anesthetized living mice. The eWATs were exposed carefully by surgery and spot injected with AAV-STEAP4 and AAV-GFP (as a control). The protein expression level of STEAP4 was examined by Western blotting. The results showed that STEAP4 was overexpressed in the eWAT but not in the inguinal white adipose tissue (iWAT) and liver of C57BL/6 mice (Figure 3).

LIMITATIONS

Adequate training and skill in performing the surgery will avoid the risk of significant damage, as much as possible. However, the surgery itself may result in some alterations, for instance, immune cell recruitment to the site where the AAV spot injection was performed.

TROUBLESHOOTING

Problem 1
Death of mouse (related to step-by-step method details).

Potential solution

- Take care of the dosage of anesthetics to avoid overdosage.
- Make moderate incisions to minimize injury.
- Ensure the person performing the surgery has adequate training and skill to reduce operation time.
- Pay attention to post-operative care.
Problem 2
Wound infection in mice caused by the surgery (related to step-by-step method details).

Potential solution

- Operate in a sterile environment.
- Ensure the person performing the surgery has adequate training and skill.
- (Optional) To prevent infection, antibiotics may be administered externally to the wound.

Problem 3
Difficulty locating and injecting the eWAT (related to AAV9 spot injection).

Potential solution

- Familiarize yourself with the anatomy of mouse abdominal cavity before operation.
- Avoid using too young mice (<= 6 weeks), as the eWAT may be too small and difficult to locate.

Problem 4
The exogenous protein expression is not limited in the eWAT (related to AAV9 spot injection).

Potential solution

- Control the volume of AAV (<= 10 μL) at each injection site by determining the required volume according to the size of the eWAT in a pre-experiment.
- Keep the sterile BD insulin syringe inside the visceral fat tissue until the injection is complete and inject the AAV slowly to avoid AAV leaking into the abdominal cavity of the mouse.
- (Optional) Add an adipose-specific promoter to the AVV vector construct.

Problem 5
The expression level of exogenous protein is low in the eWAT (related to identification the effect of spot injection of AAV).

Potential solution

- Select the appropriate serotype AAV, such as AAV9.
- Perform a pre-experiment, to ensure the exogenous protein can be expressed and detected in the white adipose tissue post AAV injection.

RESOURCE AVAILABILITY

Lead contact
Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Xiao-jun Liu (xiaojunliu@ibms.pumc.edu.cn).

Materials availability
This study did not generate new unique reagents.

Data and code availability
This study did not generate any unique datasets or code. The published article includes all datasets/code generated or analyzed during this study (Zhao et al., 2022).

SUPPLEMENTAL INFORMATION
Supplemental information can be found online at https://doi.org/10.1016/j.xpro.2022.101820.
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AUTHOR CONTRIBUTIONS
Investigation and methodology, W.Z., R.R., Q.X., X.H.X., and J.H.Y.; visualization and data curation, W.Z., R.R., and X.H.X.; writing, W.Z.; resources, H.Y. and F.D.F.; supervision and project administration, Q.X. and X.J.L.

DECLARATION OF INTERESTS
The authors declare no competing interests.

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