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Mitochondria-Targeted Antiaging Gene Therapy with Adeno-associated Viral Vectors

Dejia Li[1] and **Dongsheng Duan**[2]

[1]Department of Occupational Environmental Health, School of Public Health, Wuhan University, Wuhan, People's Republic of China

[2]Department of Molecular Microbiology and Immunology, School of Medicine, The University of Missouri, Columbia, MO USA

Abstract

Transgenic expression of catalase in mitochondria using a transgenic strategy extends life span and prevents aging-related pathology in mice. However, transgenic overexpression is not suitable for a clinical application. Adeno-associated virus (AAV) is the most promising gene delivery vehicle. Here we outline strategies on the generation of an AAV vector expressing the mitochondria-targeted catalase gene (AV.RSV.MCAT). We also describe methods for evaluating physiological impact of AV.RSV.MCAT on muscle contractility and running performance in mice.

Keywords

AAV; Adeno-associated virus; Free radical; Mitochondria; Targeted expression; Antioxidant gene therapy; Muscle; Aging; Catalase; Treadmill

1 Introduction

Reactive oxygen species (ROS) has long been implicated in aging and various neuromuscular diseases [1, 2]. The mitochondria are the primary source and major target of cellular ROS [3, 4]. Mitochondrial oxidative stress contributes significantly to human aging and disease [5]. For this reason, strategies that target ROS scavengers to mitochondria may hold tremendous potential for extending life expectancy and life quality.

Catalase is a highly conserved housekeeping antioxidant enzyme. It dehydrates two molecules of H_2O_2 to two molecules of H_2O and one molecular of O_2. In most mammalian cells, catalase is located in the matrix of peroxisomes. Interestingly, forced expression of catalase in the mitochondria results in much more potent protection against oxidative stress in cultured cells [6–8]. The effect of mitochondria-targeted catalase (MCAT) on aging was uncovered recently by Rabinovitch and colleagues [9–12]. While transgenic overexpression of peroxisomal catalase did not prolong mouse life expectance, transgenic mice carrying the

Corresponding address: Dongsheng Duan Ph.D., Professor, Department of Molecular Microbiology and Immunology, 1 Hospital Drive, M610, Columbia, MO 65212, Phone: (573) 884-9584, Fax: (573) 882- 4287, duand@missouri.edu.

MCAT gene showed significantly increased median and maximal life span. Furthermore, aging-related pathologies were greatly mitigated. These exciting results reveal a possibility of treating aging-related diseases and potentially extending life span in human patients.

Gene therapy is originally developed to cure inherited genetic diseases by introducing a normal gene [13]. Gene delivery strategies that are initially designed for treating disease may also be utilized to improve cellular function in healthy subjects [14]. Of available gene transfer vehicles, adeno-associated virus (AAV) stands out as an extremely safe and effective vector for local or whole-body gene transfer [15]. Wild-type AAV is not associated with any known human diseases, while recombinant AAV may persist in human body for at least 10 years without causing significant safety concern [16]. We have recently demonstrated that intravascular injection of AAV-9 not only resulted in robust body-wide transduction in rodents, saturated gene transfer can also be achieved in large mammals such as dogs [17, 18]. AAV vectors thus provide an excellent platform for the translation of exciting transgenic study results to humans in the future.

Investigators have generated several catalase overexpressing viral vectors including AAV catalase virus [19, 20]. Preclinical studies of AAV catalase virus suggest that this vector is safe in muscle and also ameliorates optic neuritis in the eye [19, 21, 22]. However, based on transgenic study [9], we predict that the existing AAV catalase vector cannot prolong life span. To develop a therapeutic antiaging viral vector, we engineered a novel AAV-9 virus harboring the mitochondria-targeted catalase gene (AV.RSV.MCAT). Preliminary studies suggest that AV.RSV.MCAT is well tolerated in normal mice and systemic AV.RSV.MCAT injection does not elicit noticeable toxicity. These studies also confirmed ectopic catalase expression in the mitochondria. Most importantly, treadmill running performance is significantly enhanced in mice that received AV. RSV.MCAT treatment at the neonatal period [23].

In this protocol, we outline the procedures used in the generation and characterization of the AV.RSV.MCAT vector.

2 Materials

2.1 Generation of the AV.RSV.MCAT Vector

2.1.1 Generation of the cis-MCAT Plasmid

1. Vent DNA polymerase (New England Biolabs). This is a high-fidelity thermophilic DNA polymerase. It has a low polymerase chain reaction (PCR)-related mutation rate. Store at −20 °C.
2. T4 DNA ligase (New England Biolabs). This enzyme catalyzes the formation of a phosphodiester bond between juxtaposed 5′ phosphate and 3′ hydroxyl termini in duplex DNA. This enzyme will join blunt-end and cohesive-end DNA fragments. Store at −20 °C (*see* **Note 1**).

[1]To shorten the reaction time, ligations may be done at room temperature (20–25 °C). For cohesive (sticky) ends, use 1 μL of T4 DNA ligase in a 20 μL reaction for 30 min. For blunt ends, use 1 μL of T4 DNA ligase in a 20 μL reaction for 2 h.

3. SCS110 Competent Cells (Stratagene, LA Jolla, CA, USA). The expression cassette (including AAV-inverted repeats and the MCAT gene) can be methylated when propagated in bacterial cells. SCS110 is an *endA*$^{-}$ derivative of the JM110 strain. The SCS110 strain is ideal for preparing plasmid or phagemid DNA free of Dam or Dcm methylation so that the DNA can be restriction digested by methylation-sensitive restriction enzymes. Store at −80 °C (*see* **Note 2**).
4. The *cis* plasmid carrying the vector genome (ITR-promoter-target gene-pA-ITR) (ITR stands for inverted terminal repeat).
5. The mitochondrial-tagged human catalase cDNA (MCAT) plasmid (poCAT) can be obtained from Dr. Rabinovitch at University of Washington, Seattle, WA.
6. Reagents and equipment for standard agarose gel electrophoresis.
7. 14 ml polypropylene round-bottomed tubes (Becton Dickinson Labware, Franklin Lakes, NJ, USA).
8. S.O.C. medium (Gibco-BRL). Store at room temperature.
9. Amp selection LB agar plates (100 μg/mL, ampicillin). Store at 4 °C.
10. Plasmid Miniprep Kit.
11. Standard materials for large-scale plasmid preparation with $CsCl_2$/ethidium bromide equilibrium centrifugation.

2.1.2 Recombinant AAV-9 AV.RSV.MCAT Production

1. Adenoviral helper plasmid (pHelper provides adenoviral helper function (Stratagene, La Jolla, CA)).
2. pRep2/Cap9 helper plasmid. This plasmid encodes AAV replication proteins and AAV-9 capsid. It can be obtained from Dr. James Wilson at the University of Pennsylvania, Philadelphia, PA.
3. 2.5 M $CaCl_2$. Sterilize by filtration and store at −20 °C.
4. 2× HBS buffer: 0.3 M NaCl, 1.5 mM Na_2HPO_4, and 40 mM HEPES, pH 7.05 ± 0.05. Sterilize by filtration and store at −20 °C. As pH affects transduction efficiency, it is highly suggested to double check pH before each usage.
5. DNase I (Sigma, 11 mg protein/vial, total 33 K [kunitz] units).
6. pcis-MCAT plasmid. This plasmid contains the ubiquitous Rous sarcoma virus (RSV) promoter, the mitochondrial-tagged human catalase cDNA, and the simian virus 40 (SV40) polyadenylation sequence.
7. 0.25 % Trypsin (Gibco-BRL). Store at 4 °C.
8. 10 % Sodium deoxycholate. Store at room temperature.

[2]Cells may be concentrated by centrifuging at 800 × *g* for 10 min. Pipette 200 μL SOC medium from the bottom of the tube, and then spread the mixture with a sterile spreader. Tilt and tap the spreader to remove the last drop of cells.

9. Misonic Cell Disruptor S3000 (Misonix, NY).
10. Cell lifter (Corning Incorporated, Corning, NY, USA).
11. HEPES AAV dialysis buffer: 20 mM HEPES, and 150 mM NaCl, pH 7.8. Sterilize by filtration and store at 4 °C.
12. Dialysis tubing: 12,000 MW cutoffs (Gibco-BRL). Store at 4 °C.
13. AAV digestion buffer: 0.4 M NaOH and 20 mM EDTA. Freshly made before use.
14. Slot blot hybridization solution (5× SSC, 5× Denhardt's solution, 1 % sodium dodecyl sulfate (SDS), and 50 % formamide, add 100 μg/mL denatured salmon sperm DNA just before use).
15. Dry ice bath, dry ice, and 95 % ethanol.
16. HEK293 cells: (ATCC #CRL-1573). This is a hypotriploid human fetal kidney cell line transformed by sheared human adenovirus type 5 DNA. These cells are split 1:6 every 3 days and should not be allowed to overgrow. We recommend routinely testing the cell culture for mycoplasma contamination. Cells infected with mycoplasma generally grow much slower and do not attach to tissue culture plates well.
17. Culture medium for HEK293 cells: DMEM/10 %FBS/1 %PS. DMEM (Dulbecco's modified Eagle's medium), high glucose with L-glutamine (Gibco-BRL, Grand Island, NY, ©11965-092). Store at 4 °C; Fetal bovine serum (FBS) with a high plating efficiency (Gibco-BRL, ©26140079). Store at −20 °C; penicillin and streptomycin (PS), 100 μg/mL DMEM culture medium (Gibco-BRL, ©15140-122). Store at −20 °C.

2.2 In Vivo Evaluation of MCAT Expression

2.2.1 Local Muscle Injection

1. 2–3-day-old BL6 mice (C57BL/6ScSn-Dmdmdx/J; The Jackson Laboratory).
2. Masks and gloves.
3. Biohazardous containers.
4. Cotton swabs.
5. Individual ventilation cages.
6. Needle holders (Accurate Surgical & Scientific Instruments Corp., Westbury, NY, USA).
7. 33G gas-tight Hamilton syringe and needle (Hamilton Company Reno, NV, USA).
8. Light source at an oblique angle such as a fiber optic lamp with movable arms.
9. AAV-9 in HEPES solution at 3×10^{11} viral particles per animal. 1×10^{10} viral particles/μL.

2.2.2 Systemic Facial Vein Injection

1. 1–2-day-old BL6 mice (C57BL/6ScSn-Dmdmdx/J; The Jackson Laboratory).
2. Tattoo dye.
3. 33-Gauge gas-tight Hamilton syringe (Hamilton, Reno, NV).
4. Dissecting microscope.
5. Light source at an oblique angle such as a fiber optic lamp with movable arms.
6. AAV-9 in HEPES solution at 1×10^{12} viral particles per animal. 1×10^{10} viral particles/μL.

2.2.3 Analysis of MCAT Activity

1. 0.05 M phosphate buffer (PB), pH 7.8. Store at 4 °C.
2. 30 mM H_2O_2 in 0.05 M phosphate buffer, pH 7.8. Make the 30 mM H_2O_2 freshly for each activity assay and the 30 mM H_2O_2 can be at 4 °C or room temperature during experiment.
3. UV–Vis Spectrometer.
4. Bio-Rad protein assay kit (Bio-Rad laboratories, Hercules, CA).
5. Catalase (Sigma, St Louis, MO). This serves as positive control for catalase activity assay. Aliquots of different concentration can be stored at −20 °C.

2.2.4 Quantifying MCAT Expression by Western Blot

Preparation of Whole Muscle Lysate for Western Blot

1. Homogenization buffer: pH 7.8, 0.05 M PB containing 1 % protease inhibitor cocktail (Roche, Indianapolis, IN). Use 10 mL homogenization buffer per 1 mg wet muscle weight. Store at 4 °C. During experiment the PB can be stored at 4 °C or room temperature.
2. Desktop centrifuge (Eppendorf centrifuge, model 5417C).
3. Bio-Rad protein assay kit (Bio-Rad laboratories, Hercules, CA).
4. Motor for muscle tissue grinding.
5. Liquid nitrogen.

Muscle Mitochondria Isolation

1. Homogenization buffer:

   IBm1 – 67 mM sucrose; 50 mM Tris–HCl, pH 7.4; 50 mM KCl; 10 mM EDTA; and 5 % BSA. Adjust pH with 10 N HCl. IBm2 – 250 mM sucrose; 3 mM EGTA/Tris; and 10 mM Tris–HCl, pH 7.4. Adjust pH with 10 N HCl.
2. PBS buffer (containing 10 mM EDTA and 0.2 % trypsin).
3. Teflon tissue tearor (model 985370-395, BioSpec Products, Inc.).

4. 10 % BSA in PBS buffer.
5. Bio-Rad protein assay kit (Bio-Rad laboratories, Hercules, CA).
6. Eppendorf centrifuge, model 5417C; Eppendorf–Netheler–Hinz GmbH, Hamburg, Germany.
7. Centrifuge, Sorvall Evolution RC. Rotor, SS34.
8. Tissue tearor (model 985370-395, BioSpec Products, Inc. Bartlesville, OK).

Muscle Western Blot

1. ECL Western blotting detection reagents (Amersham Biosciences, Pittsburgh, PA).
2. PVDF immobilon-P transfer membrane (EMD Millipore Corporation, Billerica, MA).
3. TBST: 1× TBS (10 mM Tris–HCl, pH 8.0; 150 mM NaCl) with 0.1 % (v/v) Tween.
4. Coomassie staining solution: 0.25 % (w/v) Coomassie blue R250, 45 % methanol, and 10 % (v/v) glacial acetic acid in water.
5. Destaining solution: 45 % (v/v) methanol and 10 % (v/v) glacial acetic acid.
6. Stripping buffer: 75 mM Tris–HCl at pH 6.8, 100 mM DTT, and 2 % SDS (w/v).
7. Blocking buffer: 5 % dry milk powder and 0.1 % Tween-20 in TBST.
8. Equipment and buffer solutions for protein SDS–PAGE electrophoresis.
9. Anti-catalase antibody (Cat© 219010 Calbiochem, San Diego, CA, USA).
10. Rabbit polyclonal to prohibitin antibody (ab28172-100 Abcam, Cambridge, MA, USA).
11. Running gel (12.5 %).
12. Stacking gel (4.5 %).
13. Tris–SDS running buffer: 1.5 M Tris, pH 8.8; 8 mM disodium EDTA; and 0.4 % SDS.
14. Tris–SDS stacking buffer: 0.5 M Tris–HCl, pH 6.8; 8 mM disodium EDTA; and 0.4 % SDS.
15. 10 % APS (ammonium persulfate, Bio-Rad Cat © 161-0700). 100 mg APS in 1 mL of distilled water (remains stable for 24 h at room temperature and 1 week at 4 °C).
16. SDS running buffer (4 l of 5× concentrations): 200 mM Tris, pH 8.8; 9 mM disodium EDTA; and 1.9 M glycine, 0.5 % SDS.
17. SDS sample dissociation buffer: 2 % SDS; 62.5 mM Tris, pH 6.8; 10 % glycerol; 3 % β-mercaptoethanol, and a few drops of a 5 % bromophenol blue.
18. Goat anti-rabbit IgG (H&L) horseradish peroxidase-conjugated affinity-purified antibody (Chemicon International, Cat # AP132P).

2.2.5 Checking Catalase Localization by Double Immunostaining

1. DakoCytomation pen (DakoCytomation, Glostrup, Denmark).
2. KPBS: 356 μM KH_2PO_4, 1.64 mM K_2HPO_4, 160 mM NaCl.
3. Gelatin (Sigma).
4. Blocking solution for immunofluorescence (IF) staining: KPBS + 1 % goat serum.
5. DPX mounting media (BDH, VWR International Ltd, Leicestershire, UK).
6. KPBSG (KPBS + 0.2 % gelatin).
7. 2-Methylbutane (Sigma).
8. Tissue-Tek OCT (Sakura Finetek Inc., USA).
9. Iodoacetic acid (Sigma, final concentration 10 mM).
10. Alexa 488-labeled goat anti-rabbit antibody (Molecular Probes, Eugene, OR; diluted 1:200).
11. Alexa 594-conjugated goat anti-mouse antibody (Molecular Probes, Eugene, OR; diluted 1:200).
12. M.O.M kit (Vector Laboratories, Inc.).
13. Rabbit antihuman catalase antibody, IgG (Cat© 219010 Calbiochem, San Diego, CA, USA) (http://www.calbiochem. com).
14. Cytochrome C (CytC) antibody, purified mouse anti-cytochrome monoclonal antibody, Clone: 6H2.B4, and IgG1. BD Pharmingen, San Jose, CA, USA (http://www. bdbiosciences.com).
15. Confocal microscope with automatic image analysis system.

2.3 Evaluation of the Effect of Mitochondrial Catalase Expression

2.3.1 In Vitro EDL Muscle Function Assay

1. Anesthetic cocktail: 25 mg/mL ketamine, 2.5 mg/mL xylazine, and 0.5 mg/mL acepromazine dissolved in 0.9 % NaCl. Keep the cocktail away from light and store at 4 °C.
2. Heating lamp (Tensor Lighting Company, Boston, MA, USA).
3. Regular dissecting forceps and scissors (World Precision Instruments, Sarasota, FL, USA).
4. Microdissecting scissors and forceps (straight and 45° angled) (Fine Science Tools, Foster City, CA, USA).
5. Bread silk suture #4-0 (SofSilk USSC Sutures, Norwalk, CT, USA).
6. Stereo dissecting microscope (Nikon, Melville, NY, USA).
7. Ringer's buffer: 1.2 mM NaH_2PO_4, 1 mM $MgSO_4$, 4.83 mM KCl, 137 mM NaCl, 24 mM $NaHCO_3$, 2 mM $CaCl_2$, and 10 mM glucose, pH 7.4. Store at 4 °C.

8. Circulating water bath (Fisher Scientific, Waltham, MA, USA).
9. Oxygen tank containing 95 % O_2 and 5 % CO_2 (Airgas National, Charlotte, NC, USA).
10. Digital caliper (Fisher Scientific).
11. Microbalance (Fisher Scientific).
12. In vitro muscle function assay system (Aurora Scientific, Aurora, ON, Canada). The system consists of a 300B or 305B dual mode transducer that measures/controls the muscle force and length, a 701A stimulator, a 604B signal interface, and an 805A in vitro test apparatus equipped with a vertically mounted tissue organ bath.
13. A custom-made 2 in. long stainless steel hook and a custom-made 2.5 in. long stainless steel hook.
14. Dynamic muscle control (DMC) software (Aurora Scientific). The software controls the force–length transducer and the stimulator. It allows real-time acquisition of the force and length data.
15. Dynamic muscle control data analysis (DMA) software (Aurora Scientific). The software analyzes the contractile data acquired by the DMC software.

2.3.2 In Vivo Evaluation of Running Distance by Treadmill

1. Exer 3/6 treadmill system (Columbus Instruments). The system includes a treadmill with an electrical stimulus and a control unit. The control unit regulates the treadmill speed and the intensity of the electrical shock. The system allows simultaneous analysis up to six animals in separate compartments.
2. Fan.

3 Methods

3.1 Generating AV. RSV.MCAT Vectors

3.1.1 Generating Catalase cis Plasmids with the OTC Leading Sequence

1. Cut out the catalase gene and OTC leading sequence from the original plasmid and insert it into the backbone of AAV cis plasmid; catalase expression is regulated by the RSV promoter and the SV40 polyA. Use standard molecular cloning methods to generate this construct.
2. Transformation of competent cells, transform 2 μL of the ligation mixture into the competent cells. Plate transformation reactions on ampicillin LB agar plates.
3. Diagnostic digestion of the plasmid with restriction enzymes that recognize unique sites in the plasmid (*see* **Note 3**).

[3]We usually used two enzymes, one enzyme recognizes the unique site in the backbone and the other enzyme recognizes the unique site in the MCAT gene.

4. Large-scale preparation of the pcis.RSV.MCAT construct (*see* **Note 4**).

3.1.2 Recombinant AAV-9 AV.RSV.MCAT Production

1. Split 293 cells at 1:3 to 150 mm culture plates. Seed a total of 15 plates for one production. Change to fresh culture medium about 1–2 h before transfection (*see* **Note 5**).
2. When cells are ~70–80 % confluent, prepare to do the plasmid transfections (*see* **Note 6**).
3. Warm up 2× HBS and 2.5 M $CaCl_2$ at 37 °C water bath for at least 30 min (*see* **Note 7**).
4. Change medium on plates with fresh DMEM/10 %FBS/1 %PS about 1–2 h before transfection (*see* **Note 8**).
5. Preparing DNA–calcium phosphate precipitate. Co-transfect three plasmids to make AAV-9 vector. These include the *cis* plasmid, pRep2/ Cap9, and pHelper. For each vector preparation (15 × 150 mm plates), use 187.5 μg of the cis plasmid, 187.5 μg pRep2/ Cap9, and 562.5 μg pHelper (at a ratio of 1:1:3). Mix all plasmids thoroughly in 15.2 mL H_2O. Add 1.68 mL of 2.5 M $CaCl_2$ to a final concentration of 250 mM. Generate DNA–calcium phosphate precipitate by slowly dropping the DNA/$CaCl_2$ mixture to 16.8 mL of 2× HBS. In general, it takes 15–30 min room temperature incubation to form a high-quality precipitate (*see* **Note 9**).
6. Gently add the DNA–calcium phosphate precipitate to 293 cells drop by drop to the culture plates. Then mix the solution in the medium by gentle shaking and put back in the incubator (*see* **Note 10**).
7. 62–65 h after transfection, collect cells with a cell lifter. Spin down the cells in a benchtop centrifuge (2,000 × *g*, 20 min at 4 °C); add 9 mL 10 mM Tris–HCl (pH 8.0). Freeze/thaw cell lysate 8–10 times using dry ice/ethanol and a 38 °C water bath. Add 10 mM Tris–HCl (pH 8.0) to increase volume to ~18 mL, and sonicate cell lysate at the power output of 5.5 for 10 min on ice (*see* **Note 11**).
8. Digest cell lysate with DNase I at 37 °C for 45 min (we normally use half vial of Sigma DNase I for each 15 plates viral preparation). Sonicate cell lysate again at the power output of 5.5 for 7 min (on ice). Digest lysate with one-tenth volume of

[4]Prior to the large-scale preparation of the pcis.RSV.MCAT plasmid, we suggest to transfect the plasmid into 293 cells and confirm catalase expression by immunocytochemistry or Western blot.
[5]To maintain cells propagated, it is important to establish a liquid nitrogen stock at ≤50 % confluence. 293 cells grown at high confluence may lose the cell propagate ability and thus reduce the yield of AAV production.
[6]To achieve high titers, it is important that 293 cells are healthy and plated at optimal density. Cells should be passaged at 70–80 % confluence. 293 cells grown at high confluence often yield low viral titer.
[7]This step is required; DNA–phosphate precipitate will form under specific temperature.
[8]Care should be taken to avoid blowing up the cells. When exchanging solutions, gently pipette down the side of the dish and not directly onto the cells to prevent disruption of the cell monolayer.
[9]A high-quality precipitate is essential to high viral yield. We recommend routinely monitoring calcium phosphate precipitate on a coverslip using a phase contrast microscope. If there is no apparently precipitate, prepare the mixture again.
[10]Do not allow the transfection mixture to sit too long before it is added to the cells. Prolonged incubation may reduce transfection efficiency.
[11]48 h after transfection, check the cells every 12 h. If there are a lot of dead cells suspended in the medium, harvest the cells immediately. Do not let cells grow more than 72 h after transfection.

0.05 % trypsin and 10 % sodium deoxycholate for 30 min in 37 °C water bath. Clear cell lysate by spinning at 3,200 × *g* for 30 min at 4 °C. Carefully transfer supernatant to a new tube.

9. Adjust the volume to 29 mL with 10 mM Tris–HCl (pH 8.0) and add 18.2 g $CsCl_2$ (this is equal to 0.613 g/mL). The final volume should be about 32.5 mL. Incubate for 30 min at 37 °C. Shake well (invert tube several times) to dissolve all $CsCl_2$. Then keep at room temperature for 1–1.5 h. Spin at 3,200 × *g* for 30 min at 4 °C. Carefully load the supernatant into six 5 mL Beckman ultracentrifugation tubes in an SW55Ti rotor. Spin at 200,000 × *g* for 40 h at 4 °C (*see* **Note 12**).

10. Collect fractions from the bottom of the tube with a 20-G needle. Identify the viral containing fractions by slot blot (see below) (*see* **Note 13**).

11. Combine fractions with the highest viral titer and centrifuge again at 200,000 × *g* for 40 h at 4 °C. Collect fractions as described in step 6 and identify highest viral fraction by slot blot (see below). The expected yield from fifteen 150 mm plates preparation is ~1×10^{13} vg particles (*see* **Note 14**).

12. The viral stock may be stored at 4 °C for 1 month before dialysis.

13. Dialyze the viral stock in HEPES buffer (4 °C for 2 × 24 h).

14. Store the dialyzed viral at 4 °C and use it in 1 week.

15. Slot blot viral titer determination. Use duplicated sets of viral stock aliquots (1, 5, and 10 μL) and plasmid copy number controls (10^7, 10^8, 10^9, 10^{10}, 10^{11} molecules/μL) in slot blot. Denature samples in 50 μL AAV digestion buffer at 100 °C for 10 min. Then immediately chill samples on ice and bring up volume to 400 μL with digestion buffer. Load samples onto Hybond-N plus membrane with a Bio-Dot SF manifold microfiltration apparatus. After blotting, cross-link DNA to the membrane with UV irradiation. Pre-hybridize the membrane, then hybridize the membrane with a 32P-labeled transgene-specific probe in the slot blot hybridization solution. Determine the viral particle titer by comparing the intensity of the viral stock band to that of the plasmid standards.

3.2 Evaluating Catalase Expression

3.2.1 Local Muscle Injection

1. For all animal experiments, get approval from the Institute Animal Care and Use Committee and follow NIH guidelines. Two muscles have been used to evaluate AAV transduction efficiency including the tibialis anterior (TA) muscle and the extensor digitorum longus (EDL) muscle.

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[12] In this protocol, we described a $CsCl_2$ ultracentrifugation-based protocol for AAV-9 purification. However, column purification (HPLC or FPLC) methods have been developed for many different AAV serotypes. It is expected that a column purification method will become available in the near future for AAV-9 [24].

[13] We usually collect 12 tubes of virus and 11 drops of virus in every tube. According to our experiences, tubes 5, 6, 7, and 8 usually contain higher concentration of virus.

[14] To accelerate this process, one may use higher spin speed.
However, centrifugation time should be adjusted accordingly.

2. Fix the 2–3-day-old neonatal mice with tap.
3. Extend the limb with the TA muscle facing forward.
4. Insert a 33-G Hamilton needle into the middle belly of the TA muscle. Slowly inject 30 μL AAV vectors into the muscle while slowly backing out the injection needle.
5. Place mice on heating pad during recovery and monitor hourly for 5 h.

3.2.2 Systemic Facial Vein Injection

1. Turn on the electronic heat pad 10 min before the experiment.
2. Adjust the light source position so that it illuminates your microscope field. A moderate light intensity is best to visualize the vein.
3. Mix tattoo dye with virus solution (6 μL/mL virus), and fill your syringe with virus. The volume should not exceed 100 μL.
4. Position the animal in view of the microscope with your non-injecting hand, and place your index finger on the side of the mouse muzzle and your middle finger just caudal to the ear bud.
5. Identify the temporal vein. It should be visible through the skin just anterior to the ear bud and inferior to small peripheral vasculature.
6. Insert the needle into the facial vein, slowly inject 100 μL virus into the vein, and slowly back out the injection needle (*see* **Note 15**).
7. Place mice on heating pad during recovery and monitor hourly for 5 h.

3.2.3 Determining Catalase Activity

1. Turn on spectrophotometer and open catalase assay program.
2. Turn on UV light 30 min before the experiment.
3. Calculate the sample volume for 200 μg protein according to the protein concentration.
4. Add 200 μg of sample to 2 mL of PB and mix well (first take the same volume of PB out). Split sample to two 1 mL aliquot.
5. Prepare 30 mM H_2O_2 freshly (add 34 μL of 30 % H_2O_2 to 10 mL PB).
6. Put 1.5 mL of PB in quartz cuvette, and click “Blank.”
7. Read sample.

    (a) Add 0.5 mL of PB to 1 mL of the sample aliquot; the readings (at 0 and 30 s) will be Ai PB and Af PB (*see* **Note 16**).

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[15]The tattoo dye can show us if the needle is inserted in the vein.
If the virus solution is leaking around the vein, stop injection immediately and try the facial vein on the contralateral side.
[16]Since there is no H_2O_2, the reading should be the same for Ai PB and Af PB. Occasionally, difference is detected due to instability of the machine/background noise. The line should be very stable at the horizontal line during assay.

(b) Dump reaction solution and put the remaining 1 mL of sample to quartz cuvette. Then add 0.5 mL of freshly prepared 30 mM H_2O_2 to this sample in quartz cuvette (mix immediately using pipette, count 3, then "Read" the sample). The reading (at 0 and 30 s) will be Ai H_2O_2 and Af H_2O_2.

8. Calculate the catalase activity (K/g) with formula K/g = [3 ln (Ai/Af)]/VPt.

3.2.4 Quantifying Catalase Expression by Western Blot

Preparation Whole Muscle Lysate for Western Blot

1. Kill the mouse by cervical dislocation.
2. Harvest TA muscle and rinse it briefly in PB to remove the blood (if no blood there, ignore this step).
3. Put the muscle into mortar; add little liquid nitrogen to freeze the muscle. Grind it into very fine powder quickly, then add 0.3 mL PB, and grind again. Transfer the homogenate to a 1.5 mL EP tube on ice (0.2 mL PB is used for completely transfer) (total 0.3 mL PB for EDL muscle), followed by a sonication at power of 6.0 for 5 min.
4. Centrifuge at 10,000 rpm for 5 min at cold room.
5. Collect supernatant (*see* **Note 17**).

Muscle Mitochondria Isolation

1. Isolate the gastrocnemius muscle.
2. Weigh 300 mg gastrocnemius muscle and rinse the muscle in 5 mL ice-cold PBS containing 10 mM EDTA (*see* **Note 18**).
3. Mince the muscle into small pieces using scissors. The minced muscles were suspended in 5 mL of ice-cold PBS supplemented with 10 mM EDTA and 0.2 % trypsin for 30 min, and then the minced muscle were put into a 15 mL tube (*see* **Note 19**).
4. Centrifuge the muscle at 200 × *g* (1,372 rpm) for 5 min.
5. The pellet was suspended in IBm1. The ratio between tissue and isolation buffer is 1:10 (w:v, mg/μL).
6. Homogenize the muscle pellet using a tissue tearor (model 985370-395, BioSpec Products, INC. Bartlesville, OK) operated at 1,600 rpm in an Oak Ridge tube on ice, 2 × 15 s with a 30 s interval (*see* **Note 20**).
7. Centrifuge at 700 × *g* for 10 min at 4 °C.

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[17] We usually measure the catalase activity immediately after harvest. Alternatively, protein pellet can be stored at −80 °C and the catalase activity measured later.

[18] It is better to use EDTA instead of EGTA. EGTA also chelates Mg^{2+}. Mg^{2+} can influence mitochondrial function as well as the kinetics of CytC release.

[19] Precool the glassware on ice for 10 min before starting the procedure. Each step must be performed at 4 °C to minimize activation of phospholipases and proteases.

[20] Wear protective gloves while you are using the homogenizer to avoid injury if the potter breaks down.

8. Transfer the supernatant to another centrifuge tube and centrifuged at 8,000 × *g* for 10 min at 4 °C (*see* **Note 21**).
9. Resuspend pellet in 5 mL of ice-cold IBm2 and centrifuge at 8,000 × *g* for 10 min at 4 °C (*see* **Note 22**).
10. Resuspend the pellet in 0.5 mL, 50 mM, pH 7.4 PB for Western blot [19]. Determine the protein concentration by a protein assay kit (Bio-Rad). The protein concentration would be 6–10 μg/μL; store aliquot sample at −80 °C.

Catalase Western Blot

1. Thaw the protein samples on ice, and add sample dissociation (loading) buffer into protein samples at ratio of 1:1.
2. Seal the sample tubes and heat at 95 °C for 5 min.
3. Load samples and run the gel at 100 V for 1–1.5 h.
4. Rinse gel by water, then set transferring “sandwich.”
5. Transfer the proteins to the PVDF membrane with Bio-Rad model: 100 V for 1 h at 4 °C.
6. Rinse the membrane by water and put it into 20 mL blocking solution (5 % milk) for 1 h.
7. Wash the membrane by 20 mL of 0.1 % TBST, and shake 10 min for three times.
8. Add primary antibody (usually at 1:1,000), and shake at room temperature for 2 h or 4 °C overnight.
9. Wash the membrane by TBST three times, totally 15 min.
10. Add secondary antibody (usually at 1:2,000), and shake at room temperature for 1 h.
11. Wash the membrane by TBST three times, totally 15 min.
12. Incubate the membrane in equal volumes of detection reagent 1 and 2 for 1 min.
13. Drain off detection reagent and wrap blots with Saran wrap.
14. Expose and develop film as required (*see* **Note 23**).

3.2.5 Checking Catalase Localization by Double Immunostaining

1. Snap freeze freshly isolated muscle sample in liquid nitrogen-cooled 2-methylbutane in Tissue-Tek OCT. Cut 8 μm muscle cryosections.

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[21]The white foamy material near the top of the tube consists of lipids. Remove the foamy material by wiping the inside of the tube with a sheet of Kimwipes.

[22]When the supernatant is poured off, the loose upper part of the mitochondrial pellet may be detached as well. Pipette out the supernatant carefully when the mitochondrial pellet tends to pour off.

[23]There are usually two bands for catalase Western blot: one band is endogenous catalase, and the other band stands for transgenic catalase.

2. Air-dry the slide.
3. Fix with 4 % paraformaldehyde for 10 min at room temperature.
4. Wash slides with KPBS 5 min for three times.
5. Block slides with KPBS + 1 % goat serum for 30 min at room temperature.
6. Incubate with KPBSG (KPBS + 0.2 % gelatin) for 5 min.
7. Incubate with primary antibody (1:500 rabbit anti-catalase antibody and 1:400, mouse monoclonal antibody against cytochrome C) in KPBSG + 1 % goat serum for 2 h at room temperature or 4 °C overnight.
8. Pour off the primary solution and wash the slides with KPBSG + 1 % goat serum 5 min for three times.
9. Incubate with secondary antibody (Alexa 594-conjugated goat anti-mouse antibody, 1:200; Alexa 488-conjugated goat anti-rabbit antibodies, 1:200) with a dilution 1:200 in KPBSG + 1 % goat serum for 1 h at RT.
10. Pour off the secondary antibody and rinse with KPBSG + 1 % goat serum 5 min for three times.
11. Coverslip with prolong gold antifading reagent (Invitrogen).

3.3 Evaluation of Effect of Mitochondrial Catalase Expression

3.3.1 In Vitro EDL Muscle Function Assay

1. Anesthetize the mouse with intraperitoneal injection of 3 μL/g body weight of the anesthetic cocktail.
2. Fill the organ bath with the Ringer's buffer. Turn on the circulating water bath to 30 °C and start to equilibrate the Ringer's buffer with 95 % O_2 and 5 % CO_2 (*see* **Notes 24** and [25]).
3. Adjust the hook to the suitable position.
4. Turn on the dual mode level system and the simulator. Load the DMC program, and set the parameters at the dynamic muscle control and data acquisition panel as following: test duration, 3 s; update frequency, 500 Hz; sampling frequency, 1,000 Hz; input style, rise/fall; first stimulation, 1 s; last stimulation, 2 s; stimulation delay, 6 ms; and pulse width, 200 ms. Set the stimulator parameters as following: trigger mode, follow; pulse phase, biphase; current multiplier, 100×; and current adjust, 8.5.
5. Record mouse and experimental information (body weight, date of birth, gender, strain, ear tag number, project title).

[24]We strongly suggest using the freshly prepared Ringer's buffer. Discard the buffer if it is more than 2 weeks old. Some investigators have included 6–25 mM tubocurarine chloride in their Ringer's buffer. However, we found this is not necessary.
[25]The contractility and fatigability of isolated mouse muscles are affected by temperature. We found that 30 °C yielded consistent force output without inducing apparent fatigue. The Ringer's buffer should be equilibrated with 95 % O_2 and 5 % CO_2 for at least 20 min before each use. During force measurement, one should adjust oxygen valve to allow for a steady flow of gas without generating large bubbles.

6. Prepare the dissection tools such as clips and scissors. Prepare the cotton rinsed in the Ringer's buffer.

7. Position the mouse face up on the dissection board and carefully peel off the skin to expose the hind limb muscles. Constantly superfuse the exposed muscles with the Ringer's buffer.

8. Dissect the remaining skin towards the metatarsal bones to expose the distal tendon of the TA muscle. Gently peel off the fascia on the surface of the TA muscle. Cut off the extensor ligament that covers the distal TA tendon. Lift up the distal TA tendon with a pair of 45° microdissection forces and separate it from the distal EDL tendon. Cut off the TA tendon and slowly peel off the TA muscle towards the knee to expose the EDL muscle. Cut out the TA muscle from its proximal attachment near the knee (*see* **Note 26**).

9. Identify the EDL tendon and clear it from the surrounding connective tissue and fat. Tie a double square knot with a bread silk suture around the distal EDL tendon. Cut the distal tendon inferior to the suture knot. Gently pull off the EDL muscle from the distal end. Separate the EDL muscle from connective tissue and vessels beneath the muscle with a pair of microdissection scissors. Tie a double square knot around the proximal EDL tendon, and tie another double square knot using the same suture to secure the proximal EDL tendon to the distal end of the steel hook. Cut the proximal EDL tendon superior to the suture knot, and remove the intact EDL muscle. Attach the proximal end of the steel hook to the level arm of the transducer. Vertically position the EDL muscle between two platinum electrodes before securing the distal EDL tendon to a fixed clamp (*see* **Note 27**).

10. Submerge the EDL muscle in the Ringer's buffer and adjust the muscle length to generate a 1 g resting tension. Allow the muscle to equilibrate for 10 min.

11. Adjust the muscle length to generate a resting tension of 1 g. Stimulate the muscle at 4 Hz (twitch stimulation). Record muscle force. Rest for ~1 min. Repeat twitch stimulation at resting tension of 1 g for two times (*see* **Note 28**).

12. Rest muscle for 1 min. Adjust the muscle length to generate a resting tension of 1 g, and stimulate muscle at 150 Hz for three times with 1 min interval.

13. Rest muscle for 5 min. Adjust the muscle length to generate a resting tension of 1 g, and stimulate muscle with a single pulse at 4 Hz. Record the force as the absolute twitch force (*see* **Note 29**).

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[26]Take extreme care to avoid cutting the blood vessels during dissection. The rupture of the vessels at the proximal end of the TA muscle may cause bleeding, which will cover the muscle tissue. When it is bleeding, remove the blood with a thin piece of Ringer's buffer-soaked cotton.

[27]To clearly expose the proximal EDL tendon, one may need to cut open the distal biceps femoral muscle. While taking the EDL muscle out, there will be bleeding from the vessels beneath the muscle. This can be stopped with a thin piece of Ringer's buffer-soaked cotton. When mounting the EDL muscle, make sure it is positioned right in the middle of two electrodes.

[28]We usually obtain the optimal muscle length at a resting tension of 1 g.

[29]Prior to the twitch and tetanic force measurement, we usually stimulate the muscle for 0.5 s at 150 Hz three times to warm up the muscle. Rest the muscle for 1 min and adjust the muscle length to the optimal muscle length between each warm-up stimulus. This treatment stabilizes muscle and allows consistent muscle force output during subsequence measurements.

14. Rest muscle for 5 min. Adjust the muscle length to generate a resting tension of 1 g, and stimulate muscle at 50, 80, 120, and 150 Hz. Rest 1 min between stimulations. Record the forces as the absolute tetanic forces under each stimulation frequency.

15. Rest muscle for 10 min. Adjust the muscle length to generate a resting tension of 1 g, and apply 10 cycles of eccentric contraction. For each cycle, stimulate muscle at 150 Hz for 700 ms. During the last 200 ms of stimulation, stretch the muscle for 10 % Lo (0.5 Lo/s). After each stimulus, restore the muscle length to Lo at a speed of 0.5 Lo/s. Allow a 2 min rest between each cycle of eccentric contraction. Record the tetanic force of the first 500 ms stimulation for each cycle. The tetanic force of the first cycle is arbitrarily designated as 100 % (*see* **Note 30**).

16. Detach the EDL muscle from the steel hook and force transducer. Cut the proximal and distal EDL tendons at the muscle tendon junction. Blot the muscle with Kimwipes twice and record the muscle weight (*see* **Note 31**).

17. Calculate the EDL muscle cross-sectional area using the equation of cross-sectional area = (muscle mass, in gram)/[1.06 g/cm^3 × (optimal fiber length, in cm)]. 1.06 g/cm^3 is the muscle density. The optimal fiber length is calculated as 0.44 × Lo. 0.44 represents the ratio of the fiber length to the Lo of the EDL muscle.

18. Load the DMA program and open the file containing the force data. Analyze the data using the software. Export the data to an Excel file. The force value obtained from the DMA program represents the absolute force. Calculate the specific muscle force by dividing the absolute force with the cross-sectional area. Calculate the force drop after eccentric contraction with the formula of Force drop % = ($F_1 - F_n$)/F_1. F_1 is the tetanic force obtained during the first cycle. F_n is the tetanic force obtained during the *n*th cycle.

3.3.2 In Vivo Evaluation of Running Distance by Treadmill

1. Record mouse/experimental information (body weight, date of birth, gender, strain, ear tag number, project title).

2. Check to make sure there is no visible injury in the limbs and toes. Record room temperature. Set up the electric shocker at the intensity of 7 and the repetition rate of 9. Adjust the inclination of the treadmill platform with the Jiffy-Jack. Turn on the fan to the low setting and have the air blow in the same direction as the mouse is running.

3. Acclimate the mouse to the treadmill for 3 days. On day 1, place the mouse on an unmoving flat treadmill for 2 min, an unmoving 15° downhill treadmill for 5 min, a 15° downhill treadmill at the speed of 5 m/min for 15 min, and a 15° downhill treadmill at the speed of 10 m/min for 5 min (*see* **Note 32**).

[30]After two rounds of stimulation, the resting tension will be around 0.7 g. If the resting force reduces to less than 0.2 g, adjust the muscle length again to obtain a resting tension of 0.5 g.

[31]When measuring the muscle weight, make sure to remove the tendons and sutures. We recommend cut right at the position of the muscle tendon junction.

4. On day 2, place the mouse on an unmoving 15° downhill treadmill for 2 min, a 15° downhill treadmill at the speed of 5 m/min for 5 min, and a 15° downhill treadmill at the speed of 10 m/min for 10 min.
5. On day 3, perform the same type of training as on day 2 except extend the last step from 10 to 15 min.
6. On day 4, subject the mouse to a single bout of 15° downhill running starting at the speed of 10 m/min. Twenty minutes later, increase the treadmill speed 1 m/min every 2 min until the mouse is exhausted. Continuously nudge the mouse to keep it stay on the track. Exhaustion is defined as the point at which the mouse spends more than 10 s on the shocker without attempting to resume running when nudged (*see* **Note 33**).
7. Record the running time at each speed and calculate the running distance.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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[32]26. To test skeletal muscle function, we recommend using 15° downhill treadmill running. Downhill treadmill running induces damaging eccentric contraction. Hence, it can more accurately reflect skeletal muscle-specific effect.
[33]Caution should be taken when using the time spent on the electric shocker as an indicator for exhaustion. Individual animal may display different running styles. Mice that do not run willingly or do not show a consistent running style should be excluded from the study.

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