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Isolation, culture and adipogenic induction of neural crest original adipose-derived stem cells from periaortic adipose tissue

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To: *Journal of Visualized Experiments* Editors

Re: Revision of manuscript "**Isolation, culture and adipogenic induction of neural crest original adipose-derived stem cells from periaortic adipose tissue**"

Nov. 18, 2019

Dear *Journal of Visualized Experiments* Editors

Enclosed please find a revised version of our manuscript (JoVE60691) entitled "**Isolation, culture and adipogenic induction of neural crest original adipose-derived stem cells from periaortic adipose tissue**".

We appreciate the editor and the reviewers' valuable comments. According to the editor and reviewers' comments, we have repeated the experiment, replace several pictures in Figure 2,3, reorganized and edited the manuscript. Taken together, our revised manuscript is significantly improved. Attached is our answers to reviewers' comments point by point.

Thank you for your continued efforts in the editorial process.

Sincerely yours,

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TITLE:

Isolation, Culture, and Adipogenic Induction of Neural Crest Original Adipose-Derived Stem Cells from Periaortic Adipose Tissue

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KEYWORDS:

neural crest cell, Wnt-1, mouse, periaortic adipose tissue, adipose-derived stromal cells, stromal vascular fraction, cell culture, adipogenic induction

SUMMARY:

We present a protocol for the isolation, culture, and adipogenic induction of neural crest derived adipose-derived stem cells (NCADSCs) from the periaortic adipose tissue of Wnt-1 $Cre^{+/-}$;$Rosa26^{RFP/+}$ mice. The NCADSCs can be an easily accessible source of ADSCs for modeling adipogenesis or lipogenesis in vitro.

ABSTRACT:

An excessive amount of adipose tissue surrounding the blood vessels (perivascular adipose tissue, also known as PVAT) is associated with a high risk of cardiovascular disease. ADSCs derived from different adipose tissues show distinct features, and those from the PVAT have not been well characterized. In a recent study, we reported that some ADSCs in the periaortic arch adipose tissue (PAAT) descend from the neural crest cells (NCCs), a transient population of migratory cells originating from the ectoderm.

In this paper, we describe a protocol for isolating red fluorescent protein (RFP)-labeled NCCs from the PAAT of Wnt-1 $Cre^{+/-}$;$Rosa26^{RFP/+}$ mice and inducing their adipogenic differentiation in vitro. Briefly, the stromal vascular fraction (SVF) is enzymatically dissociated from the PAAT, and the RFP^{+} neural crest derived ADSCs (NCADSCs) are isolated by fluorescence activated cell sorting (FACS). The NCADSCs differentiate into both brown and white adipocytes, can be cryopreserved, and retain their adipogenic potential for ~3–5 passages. Our protocol can generate abundant ADSCs from the PVAT for modeling PVAT adipogenesis or lipogenesis in vitro. Thus, these NCADSCs can provide a valuable system for studying the molecular switches involved in PVAT differentiation.

INTRODUCTION:

The prevalence of obesity is increasing worldwide, which increases the risk of related chronic diseases, including cardiovascular disease and diabetes[1]. PVAT surrounds blood vessels and is a major source of endocrine and paracrine factors involved in vasculature function. Clinical studies show that high PVAT content is an independent risk factor of cardiovascular disease[2,3], and its pathological function depends on the phenotype of the constituent adipose-derived stem cells (ADSCs)[4].

Although ADSC cell lines like the murine 3T3-L1, 3T3-F442A, and OP9 are useful cellular models to study adipogenesis or lipogenesis[5], regulatory mechanisms for adipogenesis differ between cell lines and primary cells. The ADSCs in the stromal vascular cell fraction (SVF) isolated directly from adipose tissues and induced to differentiate into adipocytes most likely recapitulate in vivo adipogenesis and lipogenesis[6]. However, the fragility, buoyancy, and the variations in size and immunophenotypes of the ADSCs make their direct isolation challenging. In addition, the different isolation procedures can also significantly affect the phenotype and adipogenic potential ability of these cells[7], thus emphasizing the need for a protocol that maintains ADSC integrity.

Adipose tissue is typically classified as either the morphologically and functionally distinct white adipose tissue (WAT), or the brown adipose tissue (BAT)[8], which harbors distinct ADSCs[9]. While ADSCs isolated from perigonadal and inguinal subcutaneous WATs have been characterized in previous studies[9-12], less is known regarding the ADSCs from PVAT that is mainly composed of BAT[13].

In a recent study, we found that a portion of the resident ADSCs in the periaortic arch adipose

tissue (PAAT) are derived from neural crest cells (NCCs), a transient population of migratory progenitor cells that originate from the ectoderm[14,15]. Wnt1-Cre transgenic mice were used for tracing neural crest cell development[16,17]. We crossed Wnt1-Cre^{+} mice with Rosa26$^{RFP/+}$ mice to generate Wnt-1 Cre$^{+/-}$;Rosa26$^{RFP/+}$ mice, in which NCCs and their descendants are labeled with red fluorescent protein (RFP) and are easily tracked in vivo and in vitro[15]. Here, we describe a method for isolating neural crest derived ADSCs (NC-derived ADSCs, or NCADSCs) from mouse PAAT and induce the NCADSCs to differentiate into white adipocytes or brown adipocytes.

PROTOCOL:

The animal protocol has been reviewed and approved by the Animal Care Committee of Shanghai Jiao Tong University.

1. Generation of Wnt-1 Cre$^{+/-}$;Rosa26$^{RFP/+}$ mice

1.1. Cross Wnt-1 Cre$^{+/-}$ mice[16] with Rosa26$^{RFP/+}$ mice[18] to generate Wnt-1 Cre$^{+/-}$;Rosa26$^{RFP/+}$ mice. House mice under a 12 h light/dark cycle in a pathogen-free facility at 25 °C and 45% humidity until they are 4–8 weeks old.

2. Dissection of the PAAT

NOTE: See **Figure 1**.

2.1. Sterilize all surgical tools (e.g., surgical scissors, standard forceps, and microsurgical scissors and forceps) by autoclaving at 121 °C for 30 min.

2.2. Prepare the digestion medium (High glucose Dulbecco's Modified Eagle Medium [HDMEM] containing 2 mg/mL collagenase type I). Prepare the culture medium (HDMEM containing 10% fetal bovine serum [FBS] and 1% v/v penicillin-streptomycin [PS]). Sterilize using a 0.22 µm syringe filter before use.

2.3. Sterilize the cell culture reagents using UV, ethanol, filtration, or steam, as appropriate.

2.4. Prepare a Petri dish with Hanks' Balanced Saline Solution (HBSS) and a 15 mL conical tube with 10 mL of HBSS supplemented with 1% v/v of PS solution. Keep both on ice.

2.5. Anesthetize the mice[15] with isoflurane and sacrifice by cervical dislocation. Immerse the bodies in a beaker filled with 75% alcohol (200 mL) for 5 min to sterilize the skin surface.

2.6. Snip and separate the skin on the abdomen and cut along the ventral midline from the pelvis to the neck. Open the abdomen and move the liver to expose the diaphragm[19].

2.7. Cut the diaphragm and the ribs on both sides of the midline and expose the heart and lungs by peeling back the ribs.

2.8. Remove the lung and thymus and extract the PAAT along with the aorta and heart.

2.9. Cut off the aorta at the aortic root to remove the heart. Make a cut between the aortic arch and descending aorta and carefully separate the adipose tissue surrounding both structures and the left and right common carotid arteries from the posterior chest wall. Transfer the tissue to the ice-cold HBSS buffer in the Petri dish.

2.10. Using sterile forceps, remove as much of the vasculature (e.g., aorta, common carotid arteries, and other small vasculature) and fascia as possible, and transfer the adipose tissue into the 2 mL Eppendorf tubes contain 0.5 mL ice-cold HBSS buffer on ice.

3. Isolation of the SVF

Collect the PAAT of 5-6 mice into one 2 mL microcentrifuge tube containing 1 mL freshly prepared digestion medium and mince the tissue using surgical scissors in an Eppendorf tube at room temperature (RT).

3.1. Transfer the mix into 50 mL tubes containing 9 mL of the digestion medium. Homogenize the tissues by pipetting up and down with a 1 mL pipette 10x.

3.2. Incubate the tubes at 37 °C with constant shaking at 100 rpm for 30–45 min and check every 5–10 min to prevent overdigestion. This is critical to improving cell viability and yield.

NOTE: Good tissue digestion will result in a homogenous, light yellow adipose tissue that is visible to the naked eye upon gently swirling the tube.

3.3. Stop the digestion by adding 5 mL of HDMEM containing 10% FBS and 1% v/v PS at RT and mix well by pipetting.

3.4. Centrifuge the cell suspension at 500 x *g* for 5 min at RT. The SVF will be visible as a brownish pellet. Carefully aspirate the floating adipocytes and decant the remaining supernatant without disturbing the SVF. Dissolve the SVF pellet in 10 mL of culture medium and filter through a 70 µm cell strainer.

3.5. Centrifuge the cell suspension at 500 x *g* for 5 min, remove the supernatant, and gently resuspend the pellet in 5 mL of erythrocyte lysis buffer in a 15 mL conical tube for 10 min at RT.

3.6. Stop the reaction by adding 10 mL of 1x PBS containing 1% FBS. Centrifuge the cell suspension at 500 x *g* for 5 min at 4 °C, remove the supernatant, and resuspend the pellet in 10 mL of 1x PBS containing 1% FBS.

3.7. Centrifuge the cells again at 500 x *g* for 5 min at 4 °C. Remove the supernatant and resuspend the pellet in 5 mL of culture medium in a 15 mL conical tube at 4 °C.

3.8. After a final round of centrifugation (500 x *g* for 5 min at 4 °C), resuspend the pelleted cells in 5 mL of FACS buffer (PBS containing 10% FBS, 100 units/mL DNA I, and 1% v/v PS) on ice, and count the cells with a hemocytometer.

4. Isolation of NCADSCs by FACS

4.1. Set up and optimize the cell sorter following the instruction manual. Select the 100 µm nozzle, sterilize the collection tubes, install the required collection device, and set up the side streams[20].

4.2. A 561 nm yellow/green laser and optical filter 579/16 are recommended for sorting RFP^+ cells. Perform the compensation using the negative control and the single-stained positive controls. See **Figure 2A** for the gating scheme.

4.3. Filter the cells through a 40 µm strainer, centrifuge at 500 x *g* for 5 min, and resuspend the cells in 2 mL of FACS buffer at a density of 0.5–1 x 10^7/mL. Transfer the cells to clearly labeled 5 mL round bottom polystyrene tubes, and load into the sorter.

4.4. Run the experimental sample tube at 4 °C, turn on the deflection plates, and sort into a 15 mL conical tube precoated with RPMI containing 1% FBS and 1% v/v PS.

NOTE: Protect the samples from strong light to minimize RFP quenching.

5. Culture of NCADSCs

5.1. Plate the sorted cells at a density of 5,000 cells/cm^2 in a 12 well culture plate in complete culture medium and incubate at 37 °C in a humid atmosphere with 5% CO_2 for 20–24 h.

5.2. Remove the culture medium, wash the cells with prewarmed (37 °C) PBS to remove cell debris and add fresh culture medium.

5.3. Once the cells are 80–90% confluent, digest the monolayer using a 0.25% trypsin EDTA solution at 37 °C in an incubator for 3–5 min, and neutralize with 2 mL of culture medium.

5.4. Centrifuge the harvested cells for 15 min at 250 x *g* at RT, remove the supernatant, and resuspend the cells in 1 mL of culture medium. Count the cells with a hemocytometer.

5.5. Seed the cells in a 12 well culture plate at the density of 5,000 cells/cm^2.

5.6. Resuspend the remaining cells in culture medium containing 10% DMSO, freeze, and store in liquid nitrogen.

6. Adipogenic induction of NCADSCs

6.1. Induce adipogenic differentiation of the NCADSCs at 80–90% confluency and standard culture conditions[21].

6.2. For brown adipogenic induction, first treat the cultured cells with brown adipogenic induction medium (HDMEM, 10% FBS, 1% v/v PS, 0.5 mM/L IBMX, 0.1μM/L dexamethasone, 1 μM/L rosiglitazone, 10 nmol/L triiodothyronine, and 1 μg/mL insulin) for 2 days. Wash the cells with PBS 2x and replace with fresh medium (HDMEM, 10% FBS, 1% v/v PS, 1 μM/L rosiglitazone, 10 nmol/L triiodothyronine, and 1 μg/mL insulin). Change this medium every 2 days for a total of 3–5x.

6.3. For white adipogenic induction, first treat the cells with white adipogenic induction medium (HDMEM, 10% FBS, 1% v/v PS, 0.5 mM/L IBMX, 0.1 μM/L dexamethasone, and 1 μg/mL insulin) for 2 days. Wash the cells with PBS 2x and replace with fresh medium (HDMEM, 10% FBS, 1% v/v PS, and 1 μg/mL insulin). Change this medium every 2 days for a total of 3-5x.

6.4. Analyze the adipogenic cells as appropriate.

NOTE: Be gentle when washing the cells with PBS. Differentiated adipocytes can easily wash away.

REPRESENTATIVE RESULTS:

Using the protocol described above, we obtained ~0.5–1.0 x 10^6 ADSCs from 5–6 Wnt-1 $Cre^{+/-}$;$Rosa26^{RFP/+}$ mice (48 weeks old, male or female).

The flow chart of collection of PAAT from mice is presented in **Figure 1.** The morphology of the NCADSCs was similar to the ADSC from other mice adipose tissues. The cultured NCADSCs reached 80–90% confluency after 7–8 days of culture, and the NCADSCs had an expanded fibroblast-like morphology (**Figure 2B**,**C**).

To further confirm that NCADSCs had adipogenic potential, differentiation of the NCADSCs into white or brown adipocytes was induced. Oil red staining was used to detect the mature adipocytes (**Figure 2**). The NCADSCs exhibited strong adipogenic potential for both white and brown adipocytes after induction. Mature adipocytes were observed after 8 days of white or brown adipogenic induction, with over 60% of the NCADSCs showing adipogenic differentiation (**Figure 2D**,**F**,**H**). Prolonging adipogenic induction time improved the harvesting rate of mature adipocytes (data not shown). NCADSCs had greatly reduced adipogenic potential after passaging (**Figure 2E**,**G**,**H**).

Immunoblotting and quantitative real-time PCR (qRT-PCR) (see **Supplemental Materials** for primers used) proved that the expression levels of adipocyte-specific relative proteins and genes (Perilipin, PPARγ, Cebp/α) in the adipogenically differentiated NCADSCs significantly increased

after 8 days of white adipogenic induction (**Figure 3A**,**B**). The qRT-PCR results showed that the induction of adipocyte-specific genes (Perilipin, PPARγ, Cebp/α) and brown adipocyte-specific genes (Pgc1α, UCP-1, PPARα, PRDM16) significantly increased in 8 days of brown adipogenic induction of NCADSCs (**Figure 3B**,**C**, **D**).

FIGURE LEGENDS:

Figure 1: Flow chart of collection of PAAT from mice. (**A**) Anesthetize and sacrifice the Wnt-1 $Cre^{+/-}$;$Rosa26^{RFP/+}$ mice and perform longitudinal dissection of the mouse to expose heart and lungs; (**B**) Remove the lungs and thymus; (**C**) Expose PAAT, aorta arch, and heart; (**D**) Remove PVAT, aorta, and heart into precooled HBSS Buffer; (**E**) Harvest PAAT and transfer into precooled HBSS buffer. H = Heart; AA = Aorta arch; T = Thymus; L = Lung.

Figure 2: Adipogenic differentiation of NCADSCs isolated from PAAT. (**A**) General gating scheme for characterizing and sorting NCADSCs (RFP) populations. (**B**) Fluorescence microscope images show that the NCADSCs adhered and expanded after 96 h seeding on a 12 well culture plate. (**C–G**) Representative images showing that oil red O stained NCADSCs from PAAT after adipogenic induction. (**C**) Control (no induction). (**D**) Primary NCADSCs and (**E**) 3x-passaged NCADSCs after 10 days of white adipogenic induction. (**F**) Primary NCADSCs and (**G**) 3x-passaged NCADSCs after 10 days of brown adipogenic induction. (**H**) Statistical results of the oil red staining area of primary and 3x passaged NCADSCs from PAAT after 8 days of adipogenic induction. n = 6. Values are expressed as mean ± standard deviation (SD). Scale bar = 50 μm.

Figure 3: Characterization of the white and brown adipogenic induction of NCADSCs. (**A**) Immunoblot showing expression levels of adipocyte-specific proteins (Perilipin, PPARγ, Cebp/α) in the white adipogenically differentiated NCADSCs. (**B**) qRT-PCR results showing the induction of adipocyte-specific genes, Cebp/α, PPARγ, Perilipin, Fabp4 in the white and brown adipogenically differentiated NCADSCs. (**C**) qRT-PCR results showing the induction of brown adipocyte-specific genes, Pgc1α, UCP-1, PPARα, PRDM16 in the white and brown adipogenically differentiated NCADSCs. The expression levels were normalized against HPRT and measured by the Ct (ΔΔCt) method. Representative result of n = 3 independent experiments. Values are expressed as mean ± SD. Unpaired 2-tailed Student t-test was used for comparisons between the two groups. *P < 0.05.

DISCUSSION:

In this study, we present a reliable method for the isolation, culture, and adipogenic induction of NCADSCs extracted from the PVAT of Wnt-1 $Cre^{+/-}$;$Rosa26^{RFP/+}$ transgenic mice designed to produce RFP^{+} ADSCs. Previous reports show that there is no significant difference in the expression of general multipotent mesenchymal stem cells (MSCs) markers in NCADSCs and non NCADSCs[22], and that NCADSCs have a strong potential to differentiate into adipocytes in vitro[15,22,23]. Thus, the NCADSCs isolated with this protocol should be suitable for most ADSC studies.

The advantage of the present method is that the inherent fluorescent reporter in the transgenic

NCADSCs makes the isolation process simple and economical without the need for antibodies or probe-based FACS, or magnetic activated cell sorting[24]. In addition, the fluorescence intensity of RFP is stronger than FITC, which further improves the efficiency of the FACS.

The key to this protocol is the utilization of young mice. Although older and larger mice can yield a greater amount of adipose tissue, the proportion of NC-derived adipocytes in the PAAT decreases with age because the NCCs primarily contribute to the early development of PAAT[15]. Thus, the adipogenic potential of these cells declines with age. Based on our experiments, the optimal time window for NCADSC isolation in mice is 4–8 weeks.

Our method is simple, practical, and can generate abundant ADSCs for the study of PVAT adipogenesis or lipogenesis in vitro, and to test novel drugs against obesity and cardiovascular disease. Moreover, the NCADSCs of Wnt-1 $Cre^{+/-}$;$Rosa26^{RFP/+}$ mice can also be an effective in vitro system for other research fields. However, several caveats remain: First, these cells are more sensitive and fragile than immortalized adipocyte lines. Second, their high proliferation rate and adipogenic differentiation is counteracted by the fact that they tend to lose their adipogenic potential after a maximum of five passages.

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DISCLOSURES:

The authors have nothing to disclose.

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Figure 1

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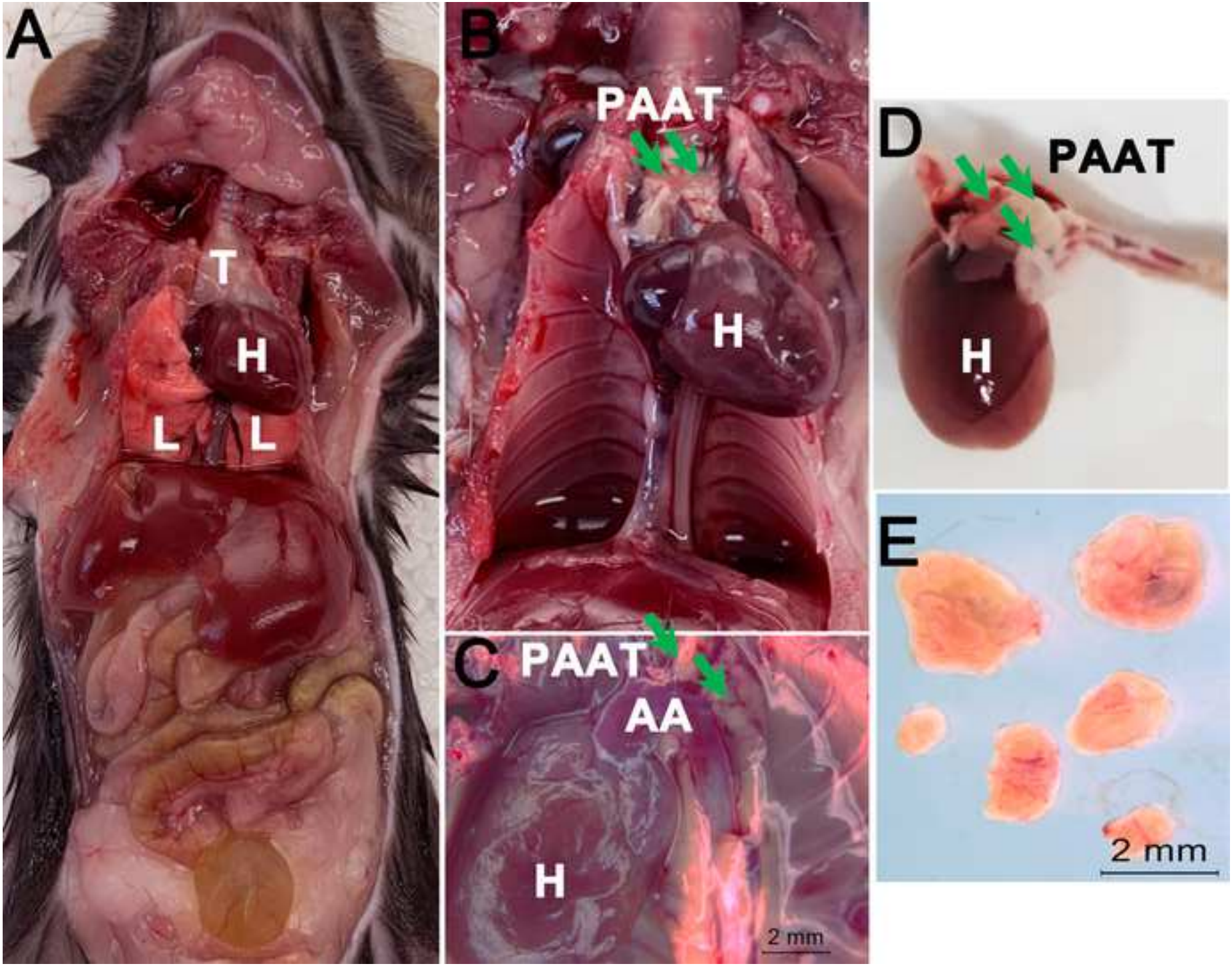

Figure 2

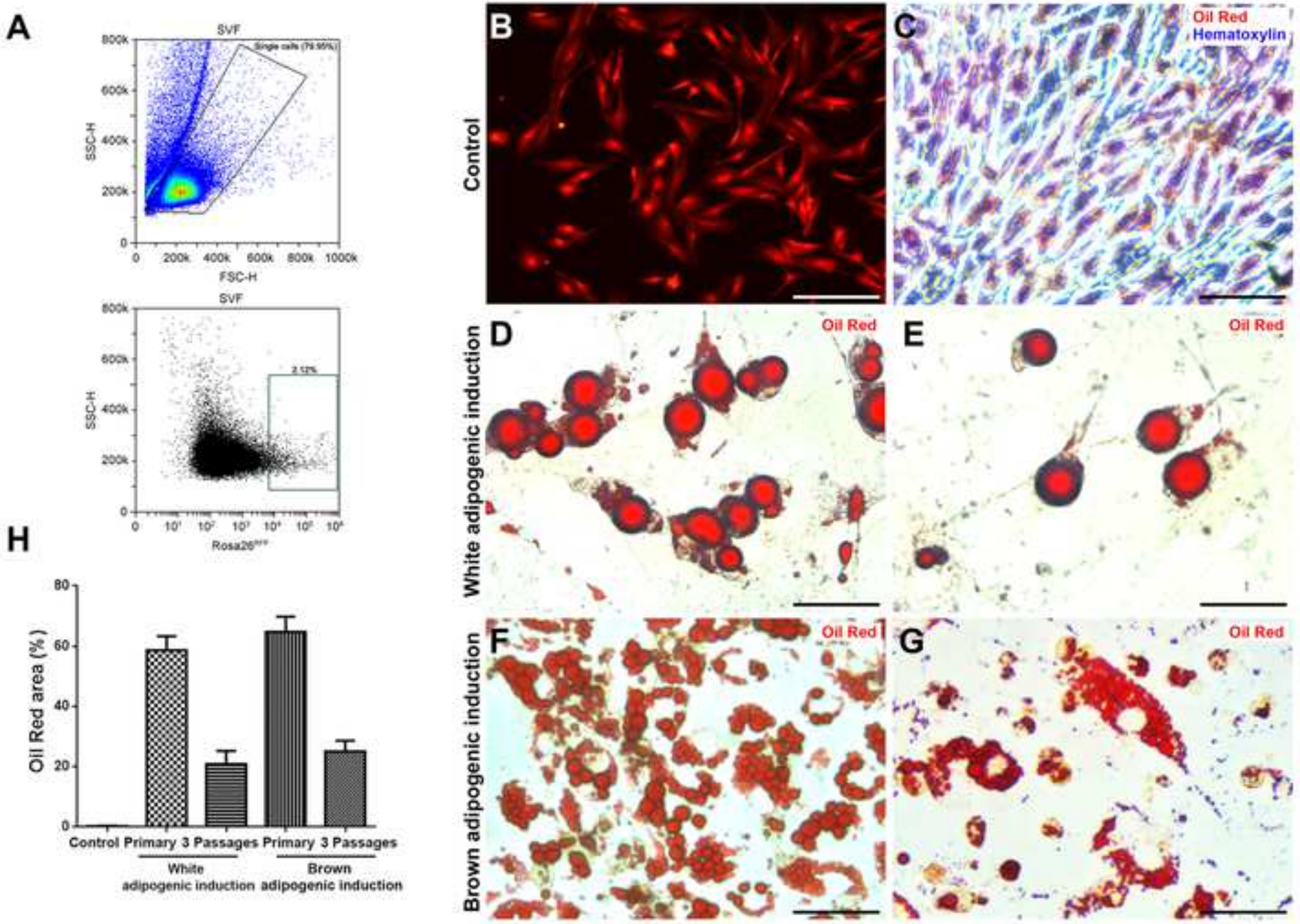

Figure 3

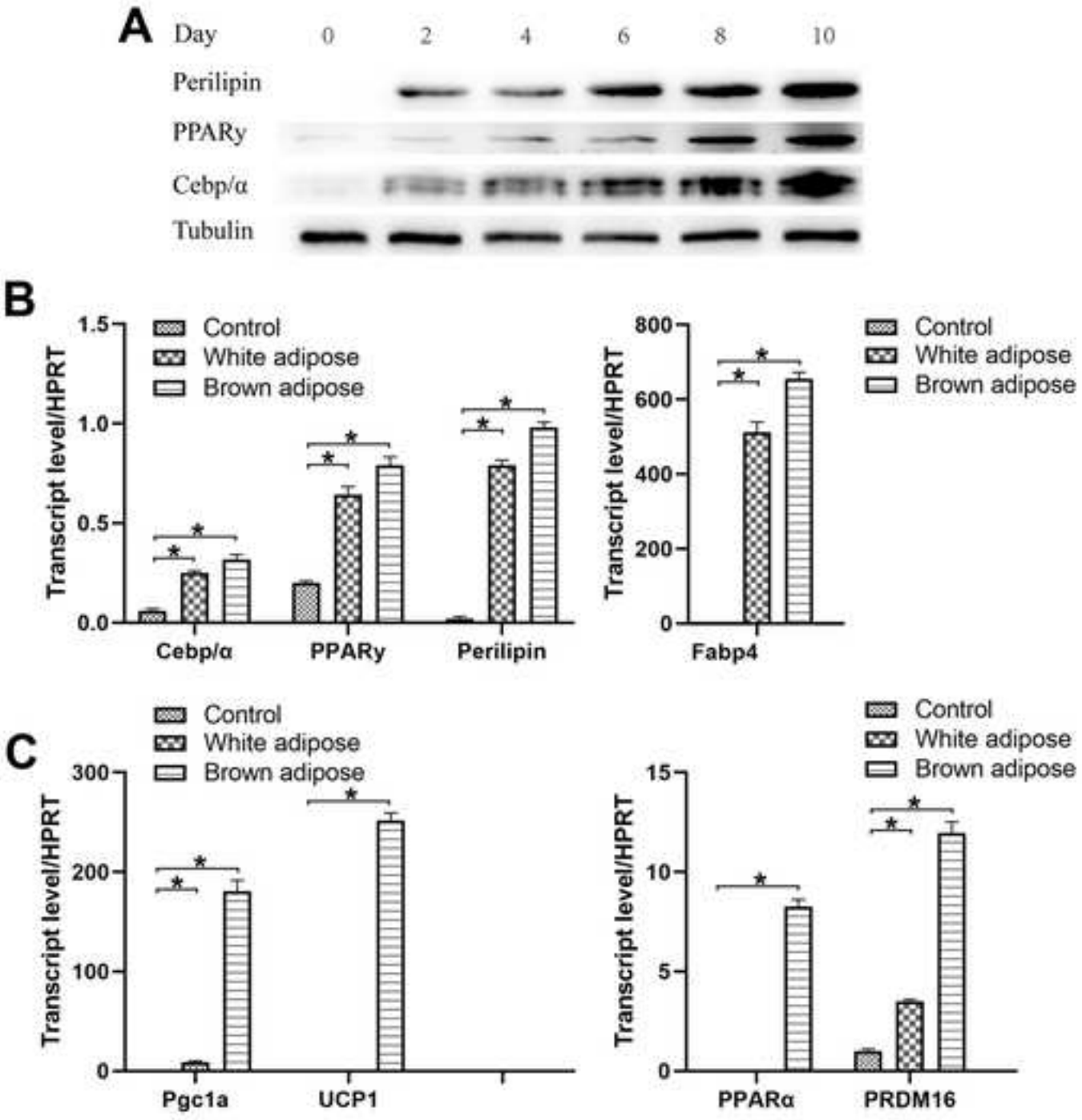

Table of Materials

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Name of Material/ Equipment	Company	Catalog Number
4% PFA	BBI life sciences	E672002-0500
Agarose	ABCONE (China)	A47902
Anti-cebp/α	ABclonal	A0904
Anti-mouse IgG, HRP-linked	CST	7076
Anti-perilipin	Abcam	AB61682
Anti-PPARγ	SANTA CRUZ	sc-7273
Anti-rabbit IgG, HRP-linked	CST	7074
Anti-β-Tubulin	CST	2146
BSA	VWR life sciences	0332-100G
Collagenase, Type I	Gibco	17018029
Dexamethasone	Sigma-Aldrich	D4902
Erythrocyte Lysis Buffer	invitrogen	00-4333
FBS	Corning	R35-076-CV
HBSS	Gibco	14025092
HDMEM	Gelifesciences	SH30243.01
IBMX	Sigma-Aldrich	I7018
Insulin	Sigma-Aldrich	I3536
Microsurgical forceps	Suzhou Mingren Medical Equipment Co.,Ltd. (China)	MR-F201A-1
Microsurgical scissor	Suzhou Mingren Medical Equipment Co.,Ltd. (China)	MR-H121A
Oil Red O solution	Sigma-Aldrich	O1516
PBS (Phosphate buffered saline)	ABCONE (China)	P41970
Penicillin-Streptomycin	Gibco	15140122
PrimeScript RT reagent Kit	TAKARA	RR047A
RNeasy kit	TAKARA	9767
$Rosa26^{RFP/+}$ mice	JAX	No.007909
Rosiglitazone	Sigma-Aldrich	R2408
Standard forceps	Suzhou Mingren Medical Equipment Co.,Ltd. (China)	MR-F424
Surgical scissor	Suzhou Mingren Medical Equipment Co.,Ltd. (China)	MR-S231
SYBR Premix Ex Taq	TAKARA	RR420A
Triiodothyronine	Sigma-Aldrich	T2877

Wnt1-Cre^{+};PPARγ$^{flox/flox}$ mice	JAX	No.009107

Comments/Description
Lot #: EC11FA0001
1% working concentration
1:1000 working concentration
1:5000 working concentration
1 µg/mL working concentration; lot #: GR66486-54
0.2 µg/mL working concentration
1:5000 working concentration
1:1000 working concentration
50 mg/mL working concentration; lot #: 0536C008
0.1 µM working concentration
50 mg/mL working concentration; lot #: R2040212FBS
Lot #: AD20813268
0.5 mM working concentration
1 µg/mL working concentration
0.3% working concentration
Lot #: AK4802
Lot #: AHF1991D
C57BL/6 backgroud; male and female
1 µM working concentration
Lot #: AK9003
10 nM working concentration

C57BL/6 backgroud; male and female

Shanghai Jiao Tong University School of Medicine
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Dear Editors and Reviewers:

Thank you for the editors' and the reviewers' comments on our manuscript entitled "**Isolation, culture and adipogenic induction of neural crest original adipose-derived stem cells from periaortic adipose tissue**". Those comments are all valuable and very helpful for revising and improving our paper, as well as the important guiding significance to our researches. We have studied comments carefully and edited the manuscript. The responds to the editor and reviewer's comments point by point are as following (the replies are highlighted in blue):

Editorial comments:

General:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

Author response: We thank the editor's advice. We carefully edited the manuscript to ensure there were no spelling and grammar mistakes.

2. Please use letter-sized paper (8.5 x 11 inches).

Author response: We used letter-sized paper (8.5 x 11 inches) for our manuscript now.

3. Please reduce the length of the Short Abstract to no more than 50 words.

Author response: We reduced the length of the Short Abstract which less than 50 words now.

4. For in-text formatting, corresponding reference numbers should appear as numbered superscripts (without brackets) after the appropriate statement(s).

Author response: We used the correct in-text formatting (JOVE formation) for references now.

5. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please limit the use of commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents.

For example: Eppendorf

Author response: We removed all trademark symbols (™), registered symbols (®), and company names in our manuscript, and all commercial products were sufficiently referenced in the "Table of Materials".

Protocol:

1. There is a 10 page limit for the Protocol, but there is a 2.75 page limit for filmable content. Please highlight 2.75 pages or less of the Protocol (including headers and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol. Remember that non-highlighted Protocol steps will remain in the manuscript, and therefore will still be available to the reader.

Author response: We highlighted in yellow of protocol text which to be featured in the video.

2. For each protocol step, please ensure you answer the "how" question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. If revisions cause a step to have more than 2-3 actions and 4 sentences per step, please split into separate steps or substeps.

Author response: We thank the editor's advice. We edited the manuscript as editor's comments. We added more references to published material specifying how to perform

the protocol action, and all steps less than 2 actions and 4 sentences in the protocol now.

Specific Protocol steps:

1. 4: Please include more details about sorting (e.g., figures with gating information).

Author response: We added a sorting picture in the Figure. 2A and including the gating information in the manuscript.

2. 5.3: Around how long will it take for cells to reach 80%-90% confluency?

Author response: It takes about 7-8 days for the cultured NCADSCs reach to 80%-90% confluency, we had mention this in the results (Page 7, Line 221).

Figures:

1. Figure 2G: What are the error bars here?

Author response: Data are presented as the mean ± standard deviation (SD). We added this illustration in the Figure legend 2.

2. Figure 3: What statistical test was use here?

Author response: Unpaired 2-tailed Student t-test was used for comparisons between 2 groups. The value of P<0. 05 was considered statistically significant. We added this illustration in the Figure legend 3.

Discussion:

1. Discussion: As we are a methods journal, please revise the Discussion to explicitly cover the following in detail in 3–6 paragraphs with citations:

a) Critical steps within the protocol

b) Any modifications and troubleshooting of the technique

Author response: We thank the editor's advice. We revised and edited the Discussion according to editor's suggestion, we added more discussion about the "Critical steps within the protocol" and "Any modifications and troubleshooting of the technique".

References:

1. Please ensure that the references appear as the following: [Lastname, F.I., LastName, F.I., LastName, F.I. Article Title. *Source*. **Volume** (Issue), FirstPage – LastPage (YEAR).] For more than 6 authors, list only the first author then et al.

2. Please do not abbreviate journal titles.

Author response: We used the correct citation formatting for references according to JOVE request.

Table of Materials:

1. Please ensure the Table of Materials has information on all materials and equipment used, especially those mentioned in the Protocol.

Author response: We edited the manuscript and listed all materials and equipment use in the Table of Materials.

Reviewers' comments:

Reviewer #1:

Manuscript Summary:

It is not correct to claim that the NCC cells differentiate more favorably to the brown lineage rather than the white. The methods documented in their recent paper indicates the authors included rosiglitazone in the brown differentiation cocktail but not in the white. It is known that rosiglitazone not only enhances differentiation it also induces the brown gene program.

Author response: We apologized for the miss descript in the introduction of the adipogenic induction medium cocktail ingredients. The rosiglitazone only used in brown adipogenic induction medium but not in the white adipogenic induction medium in our study. We revised this descript mistake in manuscript (Page 6, Line 207). And we revised discussion, we only claimed that NCADSCs easy to be induced into adipocytes in this study.

Minor Concerns:

The authors needs to provide details of how they differentiate NCC cells into white adipocytes. This is included in their recent paper but not in the methods accompanying this JOVE manuscript.

Author response: We added more detail about the method of differentiate NCC cells into white adipocytes in the manuscript (Page 6, Line 206-211)**.**

Reviewer #2:

Manuscript Summary:

The current manuscript by Qi et al. demonstrated a efficient method to isolate adipose derived stem cells from periaortic arch adipose tissues, followed by their differentiation. Despite of its potentially interesting use, there are some major flaws that need to be addressed.

Major Concerns:

1. Throughout the manuscript including the title, there is too much emphasis on neural crest cells or NCC. As the authors specified in line 52-53, only some of the ADSCs derived from NCCs. Is Wnt Cre is efficient enough to isolate only NCC derived APCs? The authors should also provide more detailed background about the mice line in the introduction.

Author response: We thank the reviewer for bringing up this very important point. Wnt1 is a well know NCCs marker and the Wnt1-Cre transgenic mouse was widely used for tracing neural crest cells development (Tamura Y. et al., 2011). According previously report (Sowa Y. et al., 2013)(Billon N., 2007) and our recently study (Fu et al., 2019), we can't exclude that Wnt1-Cre^{+} cells can differentiate into cells other than adipocytes *in vivo*, but Wnt1-Cre^{+} cells isolated from adipose tissue is efficient enough to differentiation into brown adipocytes and white adipocytes after adipogenic induction *in vitro*, which is as efficient as ADSCs purified by other cell markers.

We added more detailed background about the Wnt-1 Cre$^{+/-}$ mice (JAX Stock #009107) in "Introduction" as reviewer's suggestion (page 3, line 91).

Reference:

Tamura Y, Matsumura K, Sano M, et al: Neural crest-derived stem cells migrate and differentiate into cardiomyocytes after myocardial infarction. *Arterioscler Thromb Vasc Biol* 31:582-589, 2011.

Billon N., Iannarelli P., et al: The generation of adipocytes by the neural crest. Development (Cambridge, England) 134:2283-2292, 2007.

Sowa Y, Imura T, Numajiri T, et al: Adipose stromal cells contain phenotypically distinct adipogenic progenitors derived from neural crest. *PLoS One* 8:e84206, 2013.

Fu M, Xu L, Chen X, et al: Neural crest cells differentiate into brown adipocytes and contribute to periaortic arch adipose tissue formation. *Arterioscler Thromb Vasc Biol* 39:1629-1644, 2019.

2. Is it possible to isolate enough number of APCs and efficiently differentiate them to mature adipocytes from a single mouse? It will be more scientifically stringent than pooling APCs from multiple mice.

Author response: NCADSCs from a single mouse is not enough for one experiment. We normally pooling 5-6 mice PAAT to isolate NCADSCs in one experiment, and we explained this in the manuscript (page7, line 217).

3. Line 146, the authors should definitely inactivate RBC lysis buffer before centrifuge step.

Author response: We thank the reviewer for this valuable suggestion, and we apologize for missing this step in the protocol. We now added in (page 3, line 153).

4. Line 169. The authors should provide details of complete media composition.

Author response: The complete media composition is HDMEM containing 10% FBS and 1% v/v PS, we added this information in the manuscript (page 6, line182)

5. Line 182, generally its recommended to induce adipogenic differentiation on post-confluent pre-adipocytes. 50-60% confluency may not be optimal to start differentiation.

Author response: We thanks the reviewer bring us to this important point, and we apologize for the confusing description. Yes, contact inhibition is very important for induction of adipogenesis on ADSCs. When the cultured ADSCs reach 50-60% confluency, part of ADSCs on culture dishes begin to contact inhibition, this why we begin adipogenic induction on NCADSCs at 50-60% confluency. We had repeated the experiment as reviewer comment, adipogenic induction on NCADSCs at 80-90% confluency could increase the adipogenic induction efficiency. We had revised our protocol in the manuscript (page 6, line 196).

6. Adipogenic differentiation method need to rewritten with more details and references. In its present form its confusing.

Author response: We apologized for the confused descript in the adipogenic differentiation method. We revised the adipogenic differentiation method and added more details and reference (page6, line 198).

7.Fig. 2, picture quality of ORO is of inferior standard. Fully differentiated white adipocyte should have more bigger lipid droplets than brown adipocytes. From the ORO

pictures its not evident. **In addition, how the authors explain same levels of ORO in white vs brown adipocytes?**

Author response: We thank the reviewer bring us to this important point. Fully differentiated white adipocyte should have more bigger lipid droplets than brown adipocytes in vivo, but in cultured SVF induced adipocytes, the lipid droplet in white adipocytes only little bigger than brown adipocytes in the middle and early stage of adipogenic induction (Majka, S. M., et al., 2011). If prolonged adipogenic induction time, the difference between white and brown adipocyte lipid droplet will be bigger. We had repeated our experiment and change new picture (prolonged adipogenic induction time) in Figure 2 D-G which make our result more clear.

Because we didn't fully study the molecular mechanism of why NCADSCs is more likely to differentiate into brown adipocyte in this study, according to the reviewer's suggestion, we no longer emphasize that NCADSCs is more likely to differentiate into brown adipocyte in the manuscript.

Reference:

Majka, S. M., Barak, Y. & Klemm, D. J. Concise review: adipocyte origins: weighing the possibilities. Stem Cells. 29 (7), 1034-1040, doi:10.1002/stem.653, (2011).

8. Fig. 3, the authors should reperform all the experiments. The figure and figure legend provide no details about cell lines used. Is it white or brown adipocytes? instead of undifferentiated vs differentiated adipocytes the authors should compare white vs brown adipocytes side by side for expression of differentiation marker, lipoegenic markers and thermogenic markers.

Author response: We had repeated all experiments according reviewer’s suggestions. We now compared white vs brown adipocytes side by side for expression of differentiation marker, lipoegenic markers and thermogenic markers, the results show in Figure 3, and we revised the figure legend 3 according the change of figure 3.

Minor Concerns:

Need to add more references

Author response: We added more reference in our revision.

Reviewer #3:

Please provide detail information for each step of the procedure. it will be better if include a section of "Reagent setup" in the protocol.

Author response: We thank the reviewer for this valuable suggestion. We added more detail information for each step of the procedure as reviewer's suggest in the manuscript (Page 3, line107).

Dissection of the PAAT:

Line 104: sizes and CatLog numbers of surgical tools.

Author response: We added sizes and Cat. numbers of surgical tools in the Table of Materials.

Line 105: details about autoclave such as temperature and times; details about culture reagents.

Author response: We performed autoclave at 121 °C for 30 minutes, we now added this in the manuscript (Page3, line 105). We also listed the details about culture reagents in the manuscript (Page 3, line X107).

Line 107: information of company and CatLog of HBSS and Penicillin-Streptomycin.

Author response: We added information of company and Cat. of HBSS and Penicillin-Streptomycin in the Table of Materials.

Line 122: transfer both aorta and PAAT to chilled HBSS buffer? "chilled HBSS buffer" means ice-cold or what temperature?

Author response: "chilled HBSS buffer" means ice-cold, we revised this in the manuscript (page 4, line 127).

Line 125: detail information about the "pre-arranged tubes", such as size of tube and buffer in the tube. Will the procedure 7 be done on ice-cold condition?

Author response: We added the detail information of size of tube and buffer in the tube. The procedure 7 be done in ice-pre-cold HBSS buffer. We add all this information in the manuscript (page 4, line 130).

Line 129: company and CatLog of collagenase type I

Author response: We list the information of Cat. of collagenase type I in the “Table of Materials”.

Line 130: the procedure "mince the tissues" is not clear. Will this be done in Eppendorf tube on ice?

Author response: We "mince the tissues" by surgical scissors in Eppendorf tube at room temperature. We add this point in the manuscript (page 4, line 136).

Line 131: what is the "digestion medium"? will the procedure "homogenize" be done on ice immediately after put transfer the minced tissues into digestion medium? How many times by pipetting up and down? What's the standard?

Author response: We used HDMEM containing 2 mg/ml collagenase type I as "digestion medium". When we collect all PAAT together in the Eppendorf tube containing digestion medium, we minced tissues by surgical scissors. We homogenize the tissues by pipetting up and down with a 1 ml pipette for about 10 times. We added all this information in the manuscript (page 4, line135-139).

Line 138: what temperature (ice cold, room temperature of 37oC) of HDMEM will be?

Author response: We used HDMEM containing 10% FBS and 1% v/v PS at room temperature to stop the digestion. We added this point in the manuscript (page 4, line 145).

Line 139: "mix well" by vortex or pipetting?

Author response: "mix well" by pipetting. We added this point in the manuscript (page 4, line 146).

Line 140: "centrifuge" at 4oC or room temperature?

Author response: We Centrifuge the cell suspension at room temperature. We add this point in the manuscript (page 5, line 147).

Line 145: what is the temperature of Erythrocyte Lysis Buffer? Then directly go to procedure 6 or incubate (on ice, room temperature?) for a while?

Author response: The Erythrocyte Lysis Buffer was used to treat the re-suspend cells for 10min at room temperature. We add all this information in the manuscript (page 5, line 154).

Line 147: please provide the temperature information about centrifuge and culture medium. And the information about culture medium.

Author response: We centrifuge the cells at 4°C, the culture medium used in here is HDMEM containing 10% FBS at 4°C. We add all this information in the manuscript (page 5, line 156).

Line 149: please provide detail information of centrifugation. how cold of FACS buffer?

Author response: We centrifuge the cells at 500 g for 5 min at 4°C, the FACS was pre-cold on ice. We add all this information in the manuscript (page 5, line 163).

Culture of NC-Derived cells

Line 168 and line 176: 5000 cells/cm2 or per well?

Author response: We count density of cells by 5000 cells/cm^2.

Line 171: remove culture medium before wash the cells?

Author response: Yes, we remove culture medium, then wash the cells. We revised this in the manuscript (page 6, line 184).

Line 173: digest cells with trypsin at room temperature or 37oC in incubator?

Author response: We digest cells with trypsin at 37°C in incubator, we add this point in the manuscript (page 6, line 187).

Line 175: temperature condition of centrifuge?

Author response: We centrifuge the cells at room temperature here, we add this point in the manuscript (page 6, line 188).

Adipogenic induction of NC-Derived cells

Line 182: please make sure that induce of cell differentiation at 50-60% confluence or over-confluence.

Author response: We thanks the reviewer bring us to this important point, and we apologize for the confusing description. Yes, contact inhibition is very important for induction of adipogenesis on ADSCs. When the cultured ADSCs reach 50-60% confluency, part of ADSCs on culture dishes begin to contact inhibition, this why we begin adipogenic induction on NCADSCs at 50-60% confluency. We had repeated the experiment as reviewer comment, adipogenic induction on NCADSCs at 80-90% confluency could increase the adipogenic induction efficiency. We had revised our protocol in the manuscript (page 6, line 196).

Line 186: please provide details about "harvesting and replating the cells".

Author response: We thank the reviewer for the suggestion. We revised whole this part of protocol (page 6, line 202).

Representative results

Line 197: NC-derived ADSCs are similar to the ADSC from the other mice or depot?

Author response: We thank the reviewer bring us to this important point. We didn't study the similarity between NCADSCs and ADSC in this study, but according to previous studies (Sowa Y. et al., 2013), even NCDASCs displayed some unique antigenic features, like NCDASCs contained a higher proportion of CD24/CD34 double-positive cells, but no significant difference in the expression of general Multipotent mesenchymal stem cells (MSCs) markers between NCADSCs and non NCADSCs. We added this point in the discussion (page 8, line 271).

Reference

Sowa Y, Imura T, Numajiri T, et al: Adipose stromal cells contain phenotypically distinct adipogenic progenitors derived from neural crest. *PLoS One* 8:e84206, 2013.

Line 224: "induction" or "differentiation"?

Author response: Should be "induction" here.

Line 225: "8 days of white" or "…brown"?

Author response: We apologized for the confused descript, we mean “8 days of white or brown adipogenic induction”, we revised this in the manuscript (page 7, line 250).

Line 232: are they "brown" or "white" adipocytes?

Author response: We apologized for the confused descript, we clarified this in the manuscript (page8, line 256).

UCP1 antibody is listed in "Material", but there is no blot of UCP1 in figure. it'll be better to have it in figure.

Author response: We thank reviewer point out this, we removed UCP1 antibody in the list of "Material".

	Forward primer	Reverse primer
UCP-1	AGGCTTCCAGTACC	CTGAGTGAGGCAAA
PRDM16	CAGCACGGTGAAGC	GCGTGCATCCGCTT
Pgc1a	CCCTGCCATTGTTAA	TGCTGCTGTTCCTGT
PPARα	ACGCATGTGAAGGC	CGACAGACAGGCAC
Cebp/α	CAAGAACAGCAACG	GTCACTGGTCAACTC
PPARy	GTGCCAGTTTCGATC	GGCCAGCATCGTGT
Perilipin	AGATCCCGGCTCTTC	AGAACCTTGTCAGA
Fabp4	GGGGCCAGGCTTCT	GGAGCTGGGTTAGG
HPRT1	GTTAAGCAGTACAG	AGGGCATATCCAAC