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Isolation, Proliferation and Differentiation of Rhesus Macaque Adipose-Derived Stem Cells

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Dear Editor,

Please find enclosed our manuscript entitled "*Isolation, Proliferation and Differentiation of Rhesus Macaque Adipose-Derived Stem Cells"* that we would like to be considered for publication in *JoVE*. This paper highlights a protocol for *adipose derived stem cell isolation and differentiation.* The techniques presented in this paper and demonstrated in video format will be highly useful for researchers working in the fields of *adipose tissue research and metabolic research.*

During the preparation and submission of this manuscript, we have been kindly assisted by *Lyndsay Troyer, Ph.D.*

Thank you for your consideration of this manuscript. We look forward to hearing from you.

Sincerely yours,

Jonquil M. Poret, M.S.

TITLE:

Isolation, Proliferation and Differentiation of Rhesus Macaque Adipose-Derived Stem Cells

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KEYWORDS:

adipocyte differentiation, adipose-derived stem cells, adipocytes, rhesus macaque, adipose tissue, adipose-derived stem cell isolation, ADSC

SUMMARY:

In this article, we describe isolation of rhesus macaque derived adipose-derived stem cells (ADSCs) using an enzymatic tissue digestion protocol. Next, we describe ADSC proliferation which includes cell detachment, counting and plating. Lastly, ADSC differentiation is described using specific adipogenic inducing agents. Additionally, we describe staining techniques to confirm differentiation.

ABSTRACT:

Adipose tissue provides a rich and accessible source of multipotent stem cells, which are able to self-renew. These adipose-derived stem cells (ADSCs) provide a consistent ex vivo cellular system that are functionally like that of in vivo adipocytes. Use of ADSCs in biomedical research allows for cellular investigation of adipose tissue metabolic regulation and function. ADSC differentiation is necessary for adequate adipocyte expansion, and suboptimal differentiation is a major mechanism of adipose dysfunction. Understanding changes in ADSC differentiation is crucial to understanding the development of metabolic dysfunction and disease. The protocols described in this manuscript, when followed, will yield mature adipocytes that can be used for several in vitro functional tests to assess ADSC metabolic function, including but not limited to assays measuring glucose uptake, lipolysis, lipogenesis, and secretion. Rhesus macaques *(Macaca mulatta)* are physiologically, anatomically, and evolutionarily similar to humans and as such, their tissues and cells have been used extensively in biomedical research and for development of treatments. Here, we describe ADSC isolation using fresh subcutaneous and omental adipose tissue obtained from 4–9-year old rhesus macaques. Adipose tissue samples are enzymatically digested in collagenase followed by filtration and centrifugation to isolate ADSCs from the stromal vascular fraction. Isolated ADSCs are proliferated in stromal media followed by

approximately 14–21 days of differentiation using a cocktail of 0.5 µg/mL dexamethasone, 0.5 mM isobutyl methylxanthine, and 50 µM indomethacin in stromal media. Mature adipocytes are observed at approximately 14 days of differentiation. In this manuscript, we describe protocols for ADSC isolation, proliferation, and differentiation in vitro. Although, we have focused on ADSCs from rhesus macaque adipose tissue, these protocols can be utilized for adipose tissue obtained from other animals with minimal adjustments.

INTRODUCTION:

Adipose tissue is comprised of a heterogeneous mixture of cells, predominantly mature adipocytes and a stromal vascular fraction including fibroblasts, immune cells and adipose-derived stem cells (ADSCs)[1-3]. Primary ADSCs can be isolated directly from white adipose tissue and stimulated to differentiate into adipocytes, cartilage or bone cells[4]. ADSCs exhibit classical stem cell characteristics such as maintenance of multipotency in vitro and self-renewal; and are adherent to plastic in culture[5,6]. ADSCs are of important interest for the use in regenerative medicine due to their multipotency and ability to be easily harvested in large quantities using non-invasive techniques[7]. Adipogenic differentiation of ADSCs produces cells that functionally mimic mature adipocytes including lipid accumulation, insulin-stimulated glucose uptake, lipolysis, and adipokine secretion[8]. Their resemblance to mature adipocytes has led to the widespread use of ADSCs for physiological investigation of cellular characteristics and metabolic function of adipocytes. There are increasing evidences supporting the idea that the development of metabolic dysfunction and disorders originates at the cellular or tissue level[9-12]. Optimal ADSC differentiation is required for sufficient adipose tissue expansion, proper adipocyte function, and effective metabolic regulation[13].

Protocols described in this manuscript are straightforward techniques utilizing standard laboratory equipment and basic reagents. The manuscript first describes the protocol for the isolation of primary ADSCs from fresh adipose tissue using mechanical and enzymatic digestion. Next, the protocol for proliferation and passaging of ADSCs in stromal medium is described. Lastly, the protocol for adipogenic differentiation of ADSCs is described. Following differentiation, these cells can be used for studies to better understand adipocyte metabolism and mechanisms of dysfunction. The protocols for confirmation of adipogenic differentiation and lipid droplet detection using Oil Red O and boron-dipyrromethene (BODIPY) staining are also described. The details of these protocols focused on primary ADSCs isolated from fresh omental adipose tissue of rhesus macaques. We and others have used this protocol to successfully isolate ADSCs from rhesus macaque subcutaneous and omental adipose tissues depots[14,15]. For the same amount of tissue used, we have observed that subcutaneous adipose tissue is more dense, tougher and yields less cells from digestion compared to omental adipose tissue. This protocol has also been used to isolate ADSCs from human adipose samples[16].

PROTOCOL:

All obtained tissues and procedures were approved by the Institutional Animal Care and Use Committee at the Louisiana State University Health Sciences Center and were performed in accordance with the guidelines of the National Institute of Health (NIH publication No. 85-12, revised 1996).

1. Preparation of buffers and solutions

1.1. Prepare sterile 5-phosphate-buffered saline wash buffer (5-PBS) solution by adding 5% penicillin/streptomycin (pen/strep) and 0.25 μg/mL of fungal inhibitor to 1x PBS. Prepare sterile 2-PBS wash buffer (2-PBS) solution by adding 2% pen/strep and 0.25 μg/mL of fungal inhibitor in 1x PBS. Wash buffers can be stored at 4 ˚C for future use and must be used within 4 weeks.

NOTE: 5-PBS buffer is used for adipose tissue collection and initial washing to minimize possible contamination as tissue samples obtained at necropsy are collected in a non-sterile environment.

1.2. Prepare sterile collagenase buffer (CB) by combining 0.075% collagenase Type I (125 units/mg activity), 2% pen/strep, and 0.25 μg/mL of fungal inhibitor in Hank's balanced salt solution (HBSS) with 1% Bovine Serum Albumin. CB must be used within 1 h.

1.3. Prepare sterile ADSC growth medium using α-MEM buffer. Combine 100 mL of fetal bovine serum (FBS), 5 mL of 200 mM L-glutamine solution, 500 μL of 0.25 μg/mL of fungal inhibitor and 10 mL of pen/strep solution in 394 mL of α-MEM buffer. ADSC Growth medium can be stored at 4°C and must be used within 4 weeks.

NOTE: Heat inactivation of FBS is not necessary.

1.4. Prepare sterile ADSC differentiation medium by combining ADSC growth medium as prepared above and induction agents to achieve final concentration of 0.5 μg/mL dexamethasone, 0.5 mM isobutyl methylxanthine and 50 μM indomethacin.

2. ADSC isolation

2.1. Tissue preparation and digestion

2.1.1. Pipette ~10 mL of 5-PBS buffer into four 100 mm cell culture dishes.

2.1.2. Transfer ~50 g of adipose sample to one of the 100 mm dishes containing 5-PBS. Wash the collected adipose tissue sample four times by transferring it sequentially across the four culture dishes containing 5-PBS.

2.1.3. Transfer adipose sample to a clean 100 mm culture dish and thoroughly mince adipose tissue using scissors or two sterile scalpels. Mincing allows for increasing the surface area for efficient and complete enzymatic digestion.

2.1.4. Transfer the minced adipose tissue to 50 mL plastic conical tube containing 13 mL of CB (1–3 cm section of tissue per 15 mL of CB).

2.1.5. Rinse the culture dish with 2 mL of CB and transfer the medium to the 50 mL plastic conical tube.

NOTE: At this point, there should be a total of 15 mL of CB with tissue in a 50 mL plastic conical tube.

2.1.6. Pipette up and down several times using 25 mL serological pipette to facilitate mechanical tissue digestion.

2.1.7. Incubate the 50 mL plastic conical tube containing tissue in CB on a rocker at medium speed at 37 ˚C for 30–60 min.

2.2. ADSC plating for culture

2.2.1. Add 10 mL of ADSC Growth Medium to the 50 mL tube containing tissue to neutralize enzyme activity. Pipette up and down several times using 25 mL serological pipette to separate any adipose tissue aggregates.

2.2.2. Transfer the liquid portion to a new sterile 50 mL conical tube leaving the solids behind. Wash the original 50 mL tube 3 times with ~7 mL of 2-PBS buffer and transfer the liquid portion to the 50 mL tube.

2.2.3. Centrifuge at 500 *x g* for 5 min to obtain a cell pellet. Carefully remove as much supernatant as possible using a serological pipette without disturbing the cell pellet. Do not decant.

2.2.4. Resuspend the cell pellet in 1 mL of 1x red blood cell (RBC) lysis buffer and incubate at room temperature for 10 min. Add 5 mL of ADSC growth medium to the tube and centrifuge at 500 *x g* for 5 min, then carefully remove and discard the supernatant.

2.2.5. Add 5 mL of ADSC growth medium and centrifuge at 500 *x g* for 5 min to wash cell pellet. Discard the supernatant.

2.2.6. Resuspend the pellet in 2 mL of ADSC growth medium and filter through a 70 μm cell strainer into a new sterile 50 mL plastic conical tube.

2.2.7. Rinse the cell strainer with an additional 2 mL of ADSC growth medium. Transfer 4 mL of the suspension containing ADSCs from the 50 mL conical tube to a sterile 100 mm culture dish.

2.2.8. Wash the 50 mL conical tube 2 times with 3 mL of ADSC growth medium and transfer the liquid into the 100 mm culture dish containing ADSCs for a total of 10 mL of medium in culture dish.

2.2.9. View cells under an inverted microscope at 10x magnification to check for floating cells in the media as shown in **Figure 1A**. Cells should be maintained in a CO_2 incubator at 37 ˚C at 5% CO_2 and 100% relative humidity.

2.2.10. After 24 h, view cells under an inverted microscope to check for cell adherence. Aspirate ADSC growth medium from the plate and replace with 10 mL of fresh, warm (37 ˚C) media. Remove and replace media every 48 h until cells are 80%–90% confluent **(Figure 1B).**

NOTE: At least 10–20 % of cells will be adherent by 24 h. Check the cell culture for signs of contamination such as cell granularity, turbidity of the media, or growth of spores. Once cells are 80% confluent, they can be harvested for proliferation and cryopreservation or induced to differentiate. ADSCs can be expanded to 4–6 passages before losing their ability to efficiently proliferate or differentiate.

3. ADSC proliferation

3.1. Cell detachment

3.1.1. Remove ADSC growth medium using an aspirator/pipette. Rinse confluent ADSCs 2 times with 2 mL of sterile, room temperature PBS.

3.1.2. Aspirate PBS and add 2 mL of 0.25 % trypsin-EDTA per 100 mm culture plate. Ensure that the entire surface area is covered with trypsin.

3.1.3. Incubate plate for ~7 min at 37 ˚C in 5% CO_2 until ADSCs are detached. Remove plate from incubator and mechanically dislodge cells by forcefully pipetting the trypsin solution in the plate.

3.1.4. Add 2 mL of ADSC growth medium to the dislodged cells and gently pipette to mix. Transfer cells to sterile 50 mL plastic conical tube. To ensure maximal cell recovery, rinse culture dish with another 2 mL of ADSC growth medium, and transfer medium to the conical tube containing detached cells.

3.1.5. Centrifuge the 50 mL conical tube at 500 *x g* for 5 min at room temperature to obtain a cell pellet. Carefully decant supernatant without disturbing the cell pellet.

3.1.6. Resuspend cell pellet in 5–6 mL of ADSC growth medium per 1 x 10^6 cells.

3.2. Cell count and plating

3.2.1. In a 1.5 mL microcentrifuge tube, dilute 10 µL of cell solution from above and 10 µL of 0.04% trypan blue solution (0.4% trypan blue diluted 1:10 in PBS) and count cells using a hemocytometer.

3.2.2. Resuspend the desired number of ADSCs in growth medium. For 100 mm culture dish, plate ~3.0 x 10^5 cells in 10 mL ADSC growth media to allow for 80% confluence in 72 h or 100% confluency in 96 h.

3.2.3. View the dish under an inverted microscope at 10x magnification prior to incubation to ensure presence of suspended cells. Maintain the cells at 37 ˚C at 5% CO_2 and 100% relative humidity.

3.2.4. Every 48 h aspirate the growth medium and replace with warm (37 ˚C) medium until cells are 80% to 90% confluent as shown in **Figure 1B**.

3.2.5. Repeat steps from sections 3.1 and 3.2 for appropriate number of passages to obtain desired cell number.

NOTE: After passages 5 to 6, primary cells appear to undergo senescence; therefore, aim to obtain desired cell numbers by passage 4.

4. ADSC adipogenic differentiation

4.1. Aspirate ADSC growth medium from adhered ADSCs that have reached 80% to 90% confluence.

4.2. Quickly rinse ADSC cell layer with sterile, room temperature PBS.

4.3. Add ADSC differentiation medium. For 100 mm culture dish, add 10 mL of ADSC differentiation medium.

4.4. Replace medium every 3 days for approximately 14–21 days. Mature adipocytes are generally observed after 14 days of differentiation.

4.5. Examine cells under an inverted light microscope at 40x magnification to confirm the presence of lipid droplet as shown in **Figure 2A.**

5. Adipocyte detection

NOTE: This section will describe staining protocols used for lipid droplet detection in differentiated adipocytes, however, immunofluorescent staining for CD105, a mesenchymal stem cell marker, can also be used for ADSC confirmation.

5.1. Oil Red O staining

5.1.1. Prepare 0.5% Oil Red O stock solution in isopropanol. For 20 mL of Oil Red O stock solution, dissolve 100 mg Oil Red O in 20 mL of isopropanol. Filter the solution through a sterile syringe filter with 0.2 μm membrane.

5.1.2. Carefully aspirate the differentiation medium and wash cells 2 times by adding PBS along the sides of the well to not disturb the cell monolayer.

5.1.3. Carefully aspirate PBS from cells and add enough neutral buffered formalin (NBF, 10%) to cover cell layer. Incubate at room temperature for 30–60 min.

5.1.4. During the formalin fixation step, prepare Oil Red O working solution by diluting 3 parts of Oil Red O stock solution with 2 parts of distilled water. Mix the solution and filter through a sterile syringe filter with 0.2 μm membrane. Working solution is stable for up to 2 h.

5.1.5. Carefully aspirate NBF and quickly wash (~ 15 s) the cell layer with distilled water. Add enough 60% isopropanol to cover the cell layer after aspirating water and incubate at room temperature for 5 min.

5.1.6. Carefully aspirate isopropanol after 5 min incubation and add enough Oil Red O working solution to cover the cell layer and incubate at room temperature for 10–15 min.

5.1.7. Carefully aspirate off the Oil Red O working solution and wash the cell layer several times with distilled water until the water becomes clear.

5.1.8. Add PBS to the cell culture dish and examine cells under an inverted microscope for indication of differentiation and presence of lipid droplets **(Figure 2B).**

5.2 BODIPY Staining

5.2.1. Prepare 5 mM BODIPY stock solution by dissolving 1.3 g of BODIPY in 1 mL of DMSO. Prepare 2 μg/mL BODIPY working solution by diluting the stock solution 1:25,000 times in PBS.

5.2.2. Carefully aspirate the differentiation medium and wash cells 2 times by adding PBS along the sides of the well to not disturb the cell monolayer.

5.2.3. Carefully aspirate PBS and add enough BODIPY working solution to cover the cell layer and incubate at 37 ˚C for 30 min.

NOTE: From this point, protect cells from light by covering plate with foil.

5.2.4. Carefully aspirate the BODIPY working solution and wash the cell layer 3 times with PBS.

5.2.5. Fix cells by adding 4% paraformaldehyde and incubating at room temperature for 15 min.

5.2.6. Carefully aspirate the fixation buffer and wash cells 2 times by adding PBS along the sides of the well to not disturb the cell monolayer.

5.2.7. Add PBS to cell culture dish and examine cells under an inverted fluorescent microscope for evidence of differentiation and presence of lipid droplet **(Figure 2C).** Image cells using a FITC/EGFP/Bodipy Fl/Fluo3/DiO Chroma filter set. Excitation wavelength is at 480 nm with a band width of 40 nm and emission wavelength is at 535 nm with a bandwidth of 50 nm. A similar or appropriate filter set, and microscope can be used**.**

REPRESENTATIVE RESULTS:

The ADSCs isolated from rhesus macaque adipose tissue samples were seeded on culture plates and is shown in **Figure 1**. On the day of plating, cells are non-adherent and float in the culture dish as shown in **Figure 1A**. Within 72 h, ADSCs will become 80% confluent and are ready for adipocyte differentiation **(Figure 1B)**. ADSCs exhibit strong adipogenic characteristics after chemical induction. After 14 days of differentiation, mature adipocytes can be observed via light microscopy **(Figure 2A)**. To confirm ADSC adipogenic differentiation various stains may be used to visualize lipid droplets of the differentiated mature adipocytes. On day 14 of differentiation, cells were stained and fixed with Oil Red O **(Figure 2B)** and BODIPY **(Figure 2C)** for lipid droplet visualization. The black arrow represents a differentiated adipocyte **(Figure 2B).** These data indicate that following this protocol, ADSCs were generated from rhesus macaque adipose tissue and differentiated into mature adipocytes.

FIGURE AND TABLE LEGENDS:

Figure 1: Light micrographs of ADSCs on the day of plating and at 80% cell confluency. (**A**) Representative light micrograph of stromal vascular cells on the day of plating following ADSC isolation from fresh rhesus macaque adipose tissue (10x magnification). (**B**) Representative light micrograph of ADSCs at 80% confluency (20x magnification).

Figure 2: Micrographs of ADSCs at day 14 of differentiation. (**A**) Representative light micrograph obtained on day 14 of ADSC differentiation (40x magnification). (**B**) Representative micrograph of Oil Red O staining at day 14 of ADSC differentiation (20x magnification). (**C**) Representative micrograph of boron-dipyrromethene (BODIPY) staining at day 14 of ADSC differentiation (20x magnification).

DISCUSSION:

ADSC isolation, proliferation and differentiation protocols are straight-forward and reproducible, but they require careful technique to ensure adequate isolation, healthy expansion, and efficient differentiation. A sterile working environment is critical for all cell culture experiments. Bacteria or fungi may be introduced into cell cultures through contaminated tools, media or work environment. Fungal contamination is indicated by spore growth in the culture, while bacterial contamination is indicated by the presence of turbidity of the media. We suggest disinfecting the biosafety cabinet and all pipettes, bottles and tools before use, turning on the laminar air flow at least 15 minutes before using the biosafety cabinet and disinfecting the cabinet and all pipettes after use to reduce risk for contamination. Also, we suggest autoclaving nonfilter pipette tips for vacuum aspiration and flushing the aspirator with sterile water followed by 70% ethanol after use. A culture dish with media only can be placed in the humidified incubator at 37 °C and 5 % CO_2 for a few days to check sterility of media. Contaminated cultures should be bleached for 30

minutes and discarded. The cell culture incubator should also be disinfected.

A major difference between the protocols within this manuscript and those previously published is the use of BSA in our ADSC collagenase digestion buffer which allows for isolation of mature adipocytes and ADSCs. Additionally, our protocol utilizes ADSC growth medium supplemented with 20% FBS compared to 10% FBS as suggested by most protocols. We have noticed that rhesus macaque primary ADSCs proliferate and differentiate better with 20% FBS. ADSC differentiation in vitro is induced by lineage-specific induction agents. The induction agents used in this protocol are widely accepted for in vitro adipogenic differentiation of ADSCs. However, unlike many previously published protocols, the adipogenic differentiation cocktail does not include insulin or PPARγ agonists. We and others have used this protocol to successfully differentiate both rhesus macaque and human ADSCs[14-16].

In this protocol, ADSC adipogenic differentiation is confirmed by lipid droplet detection using Oil Red O and BODIPY staining techniques. Though these stains are well recognized in the field, there are other methods commonly used including flow cytometry for detection of CD105 to identify ADSCs, or markers of endothelial and immune cells to establish purity of ADSCs[17].

Use of ADSCs provides a valuable tool for regenerative medicine and metabolic research. ADSC isolation and proliferation produces a large number of stem cells that can be used for several downstream applications and experimental techniques including but not limited to those described in this protocol. A major intended use for differentiated adipocytes in this protocol includes the use of metabolic assays such as insulin-stimulated glucose uptake, lipogenesis, and stimulated lipolysis[18]. In addition, ADSCs can be differentiated into osteogenic and chondrogenic lineage and used for downstream analysis including for tissue engineering[7].

ACKNOWLEDGMENTS:
The authors would like to thank Curtis Vande Stouwe for his technical assistance. The research underlying development of the protocols was supported by grants from the National Institute on Alcohol Abuse and Alcoholism (5P60AA009803-25, 5T32AA007577-20 and 1F31AA028459-01).

DISCLOSURES:
None

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Figure 1

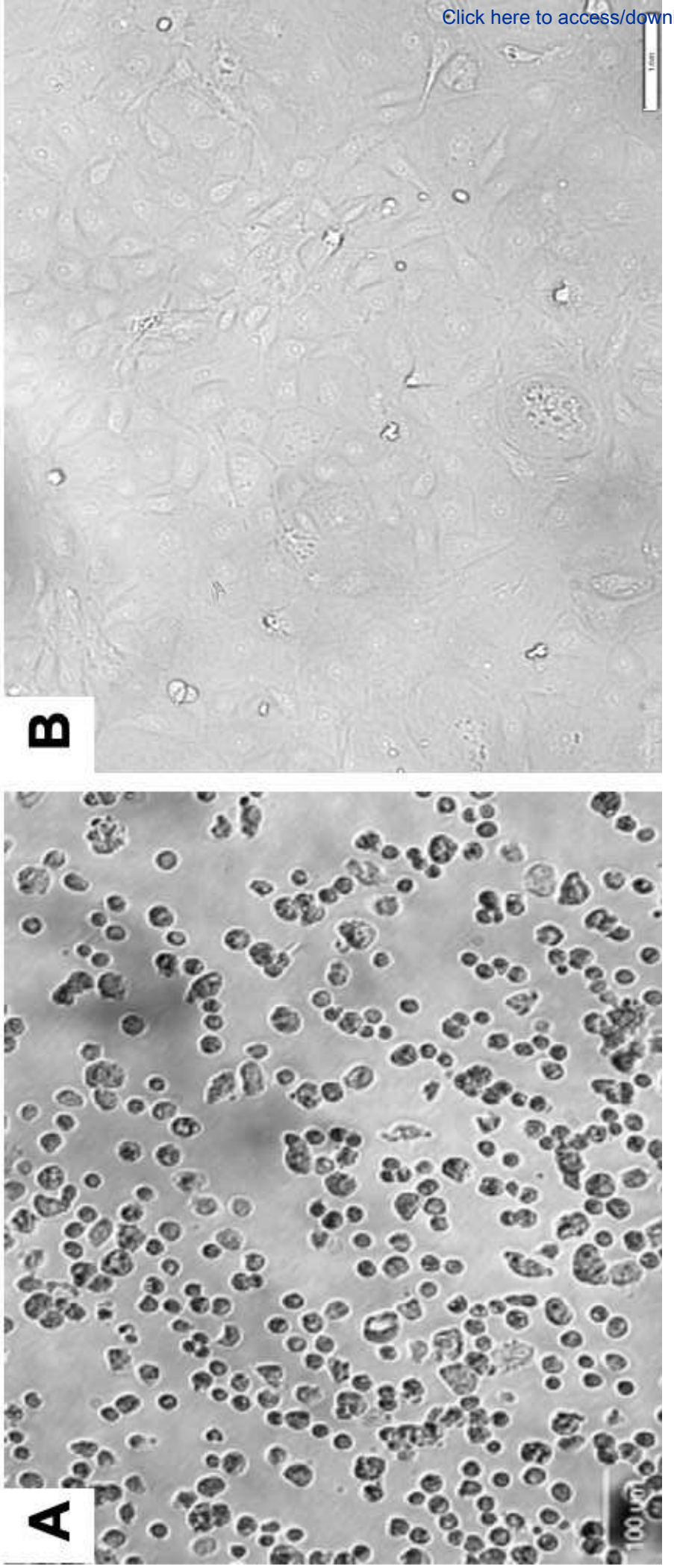

Figure 2

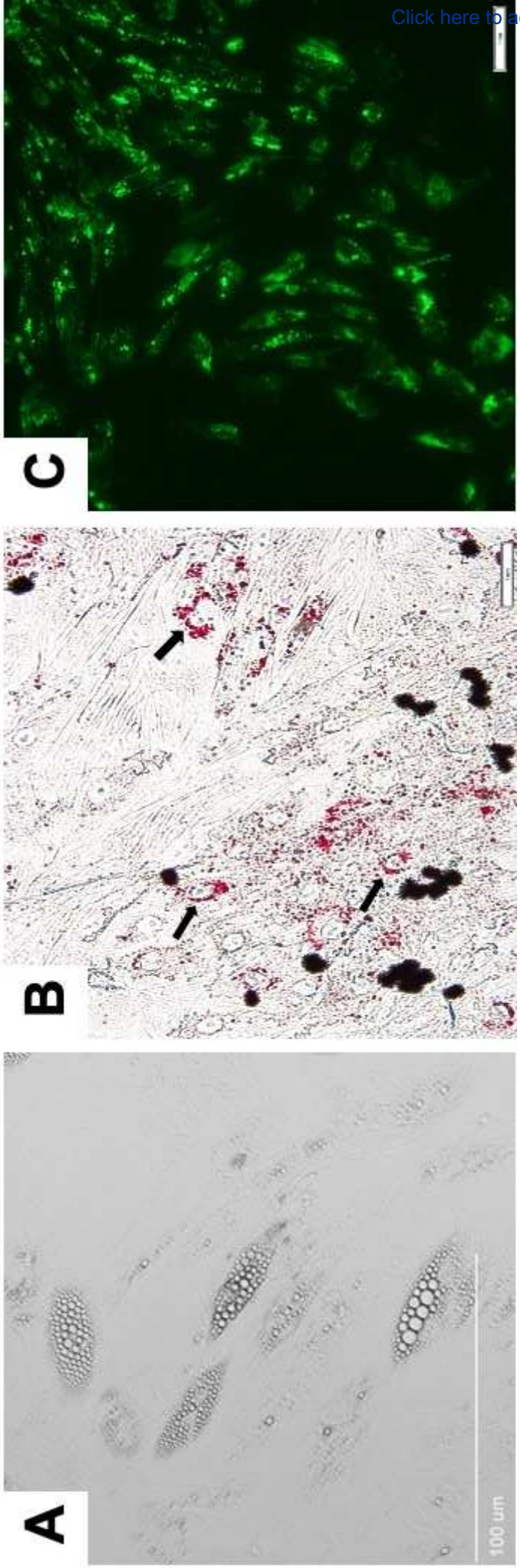

Table of Materials

Click here to access/download;Table of Materials;JoVE 61732_Materials.xlsx

Name of Material/ Equipment	Company	Catalog Number
0.4 % trypan blue	Thermo-Fisher	15250061
1.5-ml microcentrifuge tube	Dot Scientific	707-FTG
100 % isopropanol	Sigma-Aldrich	PX1838-P
100-mm cell culture dish	Corning	430167
3-Isobutyl-1-methylxanthine	Sigma-Aldrich	I7018
50-mL plastic conical tube	Fisher Scientific	50-465-232
70-µm cell strainer	Corning	CLS431751
a-MEM	Thermo-Fisher	12561056
Aluminum foil	Reynolds Wrap	
BODIPY	Thermo-Fisher	D3922
Bovine serum albumin (BSA)	Sigma-Aldrich	05470
Centrifuge	Eppendorf	5810 R
Collagenase, Type I	Thermo-Fisher	17100017
Dexamethasone-Water Soluble	Sigma-Aldrich	D2915
Dimethyl sulfoxide, DMSO	Sigma-Aldrich	D2650
Distilled water	Thermo-Fisher	15230162
Fetal Bovine Serum, USDA-approved	Sigma-Aldrich	F0926
Fungizone/Amphotericin B (250 ug/mL)	Thermo-Fisher	15290018
Hanks' Balanced Salt Solution (HBSS)	Thermo-Fisher	14175095
Hemacytometer with cover slip	Sigma-Aldrich	Z359629
Indomethacin	Sigma-Aldrich	I7378
Inverted light microscope	Nikon	DIAPHOT-TMD
L-glutamine (200 mM)	Thermo-Fisher	25030081
Laboratory rocker, 0.5 to 1.0 Hz	Reliable Scientific	Model 55 Rocking
Neutral buffered formalin (10 %)	Pharmco	8BUFF-FORM
Oil Red O	Sigma-Aldrich	O0625
Paraformeldehyde	Sigma-Aldrich	P6148
Penicillin-Streptomycin (10,000 U/mL)	Thermo-Fisher	15140122
Phosphate buffered saline (PBS), pH 7.4	Thermo-Fisher	10010023
Red blood cell (RBC) lysis buffer	Qiagen	158904

Serological pipettes, 2 to 25 mL	Costar Stripettes	
Standard humidified cell culture incubator, 37 °C, 5 % C(	Sanyo	MCO-17AIC
Trypsin-EDTA (0.25%)	Thermo-Fisher	25200056

LSU Health New Orleans
HEALTH SCIENCES CENTER
School of Medicine • Department of Physiology

August 12, 2020

Dear Dr. Nguyen,

We are pleased to submit our revised manuscript JoVE 61732 “Isolation, Proliferation and Differentiation of Rhesus Macaque Adipose-Derived Stem Cells” for publication in the Journal of Visualized Experiments. We appreciate the time and effort that the editor and the reviewers dedicated to providing feedback and are grateful for the critiques and have edited the manuscript according to the comments. We believe that our manuscript has been strengthened by the changes requested and we appreciate the opportunity for revision.

The details of the changes made are as follows. **The changes are highlighted in the manuscript. All page numbers, text lines and figures refer to the revised manuscript file with tracked changes.**

Editorial Comments:

COMMENT #1: Significant portions show overlap with previously published work. Please re-write lines 254-269, 334-338 to avoid this overlap.

We appreciate the comment, we have now rephrased the mentioned lines and have gone through the text to ensure that there is minimal overlap with published work.

COMMENT #2: Please ensure that all text in the protocol section is written in the imperative voice/tense as if you are telling someone how to do the technique (i.e. “Do this”, “Measure that” etc.) 1) Some examples NOT in the imperative: 4.5. 2) Avoid long descriptions such as on lines 78-90.

We have rephrased the protocol section to be in the imperative tense. We have also edited the text to avoid long sentences and descriptions.

COMMENT #3: Include magnification for steps 2.2.9 and 3.2.3 and mention all settings for step 4.5.

We apologize for the oversight. We have now added the Magnification for microscope objectives in steps 2.2.9 (line 174) and 3.2.3 (line 220) and settings for step 4.5 (line 245).

COMMENT #4: JoVE articles are focused on the methods and the protocol, thus the discussion should be similarly focused. Please ensure that the discussion covers the following in detail and in paragraph form (3-6 paragraphs): 1) modifications and troubleshooting, 2) limitations of the technique, 3) significance with respect to existing methods, 4) future applications and 5) critical steps within the protocol.

We have restructured the discussion to describe the 5 major points as instructed.

COMMENT #5: Fig 1-2: Add scale bars.

We apologize for the oversight; we have now added scale bars to Figures 1 and 2.

COMMENT #6: Please spell out journal names.

We have edited the references to reflect the JoVE style of citations.

COMMENT #7: Please sort table of materials in alphabetical order.

We have now sorted the Table of Materials in alphabetical order.

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Comments from Peer-Reviewers:

Reviewer #1:

COMMENT #1: I recommend using English editing service because there are multiple grammatical errors found in the text.

We have thoroughly gone through the text and ensured that there are no grammatical or textual errors. We apologize for the typographical mistakes.

COMMENT #2: In my experience, the process human ADSCs isolation from visceral fat (collagenase digestion/centrifugation/separation of adipocytes) produces 1st SVF that after few days in culture in regular medium (DMEM/10% FCS/antibiotics) contains ~50% ADSCs and ~ 50% endothelial cells distinguished by their own morphology. The authors use 20% FCS, that it is ok, although most of the protocols indicate the use of 10%FCS.

Following collagenase digestion and separation of adipocytes and RBC lysis, the SVF fraction is plated in ASC media. The media is changed after the first 24 h, to remove most immune and other non-adherent cells and subsequently, media is changed every 48 h until the cells are about 80% confluent. We and others have shown using this protocol that most adherent cells have a fibroblast morphology and differentiate efficiently into adipocytes. Although we cannot unequivocally prove, the cultured SVF fraction is free of most of the contaminating cell types including RBCs, immune cells, and cells of endothelial lineage. We were using 10% FBS initially as suggested by most protocols but based on studies we have performed with primary macaque ADSCs, the cells proliferate and differentiate better in 20% FBS. Hence, for primary macaque ADSCs we recommend 20% FBS be used.

COMMENT #3: The preparation of collagenase digestion buffer (CB) does not require 1% BSA. If BSA is added to CB it is necessary adjust the PH to 7.4, because BSA make CB acidic. If 1% BSA is present in CB, I do not recommend to keep it at 4C for 4 weeks, because the possibility of bacterial infection, without BSA it is OK.

BSA is added in the collagenase digestion buffer to allow for separation of mature adipocytes during the digestion process. These mature adipocytes can then be used for downstream analysis. As mentioned in the protocol (lines 100-102), we recommend that CB be made fresh during the day of isolation and be used within 1 hour.

COMMENT #4: Besides dex, IBMX, adipogenic medium require the presence of insulin and PPARg agonist. CRITICAL

We and others have successfully differentiated both rhesus macaque and human adipose samples without insulin and PPARγ agonists in adipogenic medium (Ford, SM *et al.* 2018; Bunnell, BA *et al.* 2008; Gagliardi, C & Bunnell, BA 2011). In our laboratory, ADSCs are primarily used for metabolic studies involving assays for insulin-stimulated responses. To reduce the potential for insulin used in the differentiation cocktail to affect adipocyte metabolic capacity, and because the cocktail we use allows for optimal adipogenic differentiation, we recommend using the cocktail without insulin, especially for studies that will use differentiated adipocytes for downstream insulin-stimulated assays.

COMMENT #5: If cells are not adherent after 48 hs (line, 178) mean all cells are death, it is irrelevant to increase concentration of FBS in the ADSC growth medium.

We agree with the reviewer's comment. We have rephrased the sentence to read that after 24 hours at least 10-20 % of cells should be adherent and if there are no adherent cells, it indicates cell death due

to unforeseen issues during the isolation process or contamination of cell cultures (lines 183-187).

COMMENT #6: For 300,000 cells, I recommend to resuspend them in 5 cc culture medium and plate in 60 mm dish.

With primary ADSCs, one of the objectives is to have maximal number of cells for all proposed experiments. We have performed a number of optimization experiments for seeding densities and have found that if 300,000 cells are plated on a 100 mm culture dish, we can achieve ~100% confluency in 96 hours and it does not affect the gene expression or differentiation potential of ADSCs. Hence, we are fairly confident in recommending 300,000 cells in 100mm cell culture plates for expanding primary macaque ADSC cell lines.

COMMENT #7: Authors should describe concs of Oil Red O and BODIPY used, time of incubations, washes, etc.

We apologize for the oversight. The protocol is now updated to include all relevant information for Oil Red O and BODIPY staining.

COMMENT #8: I also recommend to characterize ADSCs using immunofluorescent label CD105.

We thank the reviewer for the suggestion. We use Oil Red O staining, BODIPY immunofluorescent staining and adipogenic gene expression to determine differentiation potential of ADSCs. CD105 is a good marker to confirm that the cells isolated are indeed adipose derived mesenchymal stem cells, although it is not routinely done in our laboratory. However, we have now added that CD105 can be used to confirm ADSCs in the Note section (lines 249-251).

Reviewer#2? (comments in PDF document)

COMMENT #1: plastic adherence is not a feature of multipotency

We have rephrased the sentence to describe the characteristics of stem cells and the adherence properties of ADSCs (lines 56-58).

COMMENT #2: how similar? please indicate the differences

We have rephrased the sentence to read “For the same amount of tissue used, we have observed that subcutaneous adipose tissue is more dense, tougher and yields less cells from digestion compared to omental adipose tissue ” (lines 79-81).

COMMENT #3: identification of lipid vesicles

We have now replaced “vesicles” with “droplets” where appropriate.

COMMENT #4: Please, explain the use of 5-PBS vs 2-PBS wash buffers

Because necropsy and collection of adipose tissue from macaques are performed in a non–sterile environment, we use higher concentrations of antibiotics to minimize risk of contamination. We have now the explained the use of 2 different concentrations of antibiotics in the Note section (lines 97-98).

COMMENT #5: Vendor, Country of origin, heat inactivated?

The details of FBS is provided in the Table of Materials. This is to adhere to the manuscript guidelines.

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COMMENT #6: Indicate the weight of adipose tissue

We generally use ~50 g of adipose tissue. We have now added this to the manuscript, line 119.

COMMENT #7: Mincing does not free cells

We apologize for the error. We have rephrased the sentence to indicate that mincing allows to increase the tissue surface area for more efficient and complete enzymatic digestion (lines 124-125).

COMMENT #8: Since the tube is sealed, there is no need to keep it in 5%CO2

We agree with the reviewer, enzymatic digestion need not be performed in 5% CO2. We have revised the step (2.1.7).

COMMENT #9: RT or 37

We have clarified the step and added that room temperature PBS is used.

COMMENT #10: "Rotate"

Step 3.1.2 indicates how to add Trypsin to the plate. Because only 2 ml of trypsin is added, the sentence was added to emphasize that the whole surface area should be covered. We have rephrased the sentence to read "Aspirate PBS and add 2 mL of 0.25 % trypsin-EDTA per 100-mm culture dish. Ensure the entire plate surface area is covered with trypsin."

COMMENT #11: Please replace Fig 1B with a sharper image

We apologize and have replaced Fig 1B.

COMMENT #12: Why does nuclei appear blue?? There is no DAPI being added to the staining buffer

We agree with the reviewer and we apologize, we have now removed the sentence.

COMMENT #13: Vendor #cat

The details of BODIPY is provided in the Table of Materials. As mentioned earlier, this is to adhere to the manuscript guidelines.

COMMENT #14: Indicate the wavelength and filter sets used for imaging

We have added the details used for imaging in the text (lines 304-306).

COMMENT #15: Typographical errors

We have addressed the specific comments by the reviewer and have thoroughly gone through the text and ensured that there are no grammatical or textual errors. We apologize for the typographical mistakes.

Reviewer #3:

COMMENT#1: As mentioned by authors on line 54 "Primary ADSCs can be isolated directly from white adipose tissue and stimulated to differentiate into adipocytes, cartilage or bone cells", I would suggest that both osteogenic and chondrogenic differentiation should be performed to ensure the multipotency of the isolated cells.

We agree with the reviewer that ADSCs are multipotent. However, our laboratory's primary focus is to elucidate how adipogenic metabolic capacity of adipocytes is influenced by external stressors. Based

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on our expertise, we have focused this manuscript on isolation, proliferation and adipogenic differentiation of ADSCs primarily for metabolic studies and assays. However, we have added in the introduction that ADSCs are multipotent and can be use in studies relating to regenerative medicine.

COMMENT #2: Figure 1B should be replaced by a higher quality image since it's impossible to distinguish the morphology of the cells.

We apologize and have replaced Fig 1B.

Thank you, in advance, for your time and consideration.

Sincerely,

Liz Simon
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