Chapter 2

Isolation of Mouse Stromal Vascular Cells for Monolayer Culture

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Abstract

Positive energy balance contributes to adipose tissue expansion and dysfunction, which accounts largely for obesity and related metabolic disorders. Thermogenic fat can dissipate energy, activation or induction of which may promote energy balance and address the pressing health issues. Recent studies have shown that stromal vascular fraction (SVF) from white adipose tissue (WAT) can develop both white and brown-like adipocyte phenotypes, thus serving as a unique model to study adipogenesis and thermogenesis. Here, we describe a protocol for effective isolation of mouse SVF from WAT, induction of differentiation, and detection of adipogenesis. Success tips for isolation and culture of SVF are also discussed.

Key words Adipose tissue, Stromal vascular fraction, Adipogenesis, Lipid accumulation, Energy balance, Obesity

1 Introduction

Obesity is one of the most pressing health issues worldwide [1, 2]. Imbalance between energy intake and expenditure contributes to aberrant adiposity, which is associated with changes in mitochondrial function, hormonal signaling, redox status, and inflammatory responses [3–10]. These changes account largely for obesity-related medical conditions including diabetes, cardiovascular diseases, and cancer [2, 11, 12]. The mechanism of obesity-induced metabolic disorders is complex, but it has been increasingly recognized that adipose tissue dysfunction impairs energy expenditure (brown adipose tissue, BAT), energy storage (white adipose tissue, WAT), and endocrine regulation of metabolism via adipokines [3–6]. Thus, targeting adipocyte tissue function to promote thermogenesis for energy homeostasis, such as activation of BAT and browning of WAT, has attracted much interest [13–16].
Adipose tissue is composed of other cell types (collectively called stromal vascular fraction, SVF) in addition to adipocytes, including precursor stem cells, preadipocytes, and fibroblasts [17, 18]. Although immortalized cell lines (e.g., 3T3-L1, 3T3-F442A) provide convenient models for adipogenic study, differences in WAT marker expression and trans-differentiation potential have been recognized between these cell lines and primary cells isolated from adipose tissues [17–19]. For instance, SVF isolated from WAT can develop both white and brown-like adipocyte (brite or beige cell) phenotypes upon cold or adrenergic stimulation, and a bidirectional interconversion exists [20–22]. Therefore, isolation and use of SVF from adipose tissues is of critical importance to understand adipocyte biology. In this chapter, we provide a protocol that demonstrates effective isolation of SVF for monolayer culture and adipogenic study.

2 Materials

2.1 Isolation of SVF

1. Equipment and supplies: 50-mL sterile conical tubes, 0.2-μm filter units, scissors and forceps (autoclave to sterilize), cell strainer (40 μm), light microscope, shaking water bath, benchtop centrifuge, and biosafety cabinet.

2. Krebs–Ringer bicarbonate (KRB) buffer: 118 mM NaCl, 5 mM KCl, 2.5 mM CaCl₂, 2 mM KH₂PO₄, 2 mM MgSO₄, 25 mM NaHCO₃, 5 mM glucose, pH 7.4. KRB buffer is sterilized using 0.2-μm filter units.

3. Phosphate-buffered saline.

4. Collagenase solution: 300 U/mL collagenase (type I) in sterile KRB, with 1 % bovine serum albumin (fraction V).

5. Basal medium: DMEM/F12, 10 % Fetal Bovine Serum (FBS), and 1× Pen/Strep.

6. Mice: 8–12 week old, male C57BL6/J mice were housed as described previously, in plastic cages on a 12-h light–dark photocycle and with free access to water and regular chow diet [1]. Animal use procedures followed the National Institutes of Health guidelines and were approved by the Virginia Tech Institutional Animal Care and Use Committee.

2.2 SVF Monolayer Culture

1. Equipment and supplies: cell culture dishes and plates (collagen coated and noncoated), 0.2-μm filter units, inverted microscope, laboratory CO₂ water-jacketed incubators, benchtop centrifuge, and biosafety cabinet.

2. Phosphate-buffered saline.

3. 0.25 % Trypsin in HBSS.

4. Basal medium: DMEM/F12, 10 % FBS, and 1× Pen/Strep.
1. Equipment and supplies: inverted microscope, laboratory CO₂ water-jacketed incubators, and biosafety cabinet.

2. Differentiation medium: DMEM/F12 medium containing 10 % FBS, 1× Pen/Strep, dexamethasone (5 μM), insulin (0.5 μg/mL), IBMX (0.5 mM), and rosiglitazone (1 μM).

3. Maintenance medium: DMEM/F12 medium containing 10 % FBS, 1× Pen/Strep, and insulin (0.5 μg/mL).

1. Equipment and supplies: 0.2-μm filter units, 96-well plates, inverted microscope, microplate reader, and western blot analysis system.

2. Oil Red O.

3. Isopropanol.

4. 10 % neutral buffered formalin.

5. Phosphate-buffered saline.

6. Chemiluminescent horseradish peroxidase (HRP) substrates.

7. Primary antibodies against adiponectin.

8. Primary antibodies against PPARγ.

9. Primary antibodies against GAPDH.

10. Secondary antibodies conjugated to HRP.

11. PLC lysis buffer: 30 mM Hepes, pH 7.5, 150 mM NaCl, 10 % glycerol, 1 % Triton X–100, 1.5 mM MgCl₂, 1 mM EGTA, 10 mM NaPPi, 100 mM NaF, 1 mM Na₃VO₄, supplemented with protease inhibitor cocktail and 1 mM PMSF immediately before use [1, 23].

3 Methods

3.1 Isolation of SVF

1. Sacrifice the mice by CO₂ asphyxiation, followed by cervical dislocation as a second means of euthanasia.

2. Apply 75 % ethanol spray to the fur for sterilization.

3. To collect subcutaneous (inguinal) white adipose tissue (sWAT), remove the skin along the thighs to reveal sWAT pads on both sides; carefully dissect sWAT pads, and quickly remove any contamination (e.g., hair, muscle, or connective tissue). Store sWAT pads in sterile KRB buffer (on ice) briefly while additional samples are collected.

4. To collect visceral (epidydimal) white adipose tissue (eWAT), change gloves and use another set of clean sterile surgery tools. Cut the peritoneum to expose abdominal organs, locate the testes, and identify the attached eWAT. Carefully dissect eWAT pads from both sides, and quickly remove any contamination (e.g., hair, testes, epididymides, and vasa deferentia). Store the
eWAT pads in sterile KRB buffer (on ice) briefly while additional samples are collected.

5. Quickly dry sWAT and eWAT pads on Kim Wipe paper to remove residual KRB, and place the tissues on clean dry petri dishes.

6. Add CaCl₂ (at a final concentration of 2 mM) to freshly made collagenase solution to make digestion medium.

7. Add 10 mL digestion medium to the clean dry petri dishes where sWAT and eWAT are placed, mince the tissues into small pieces (1–2 mm).

8. Cut 2–3 mm from the end of 5 mL pipettes, and transfer the minced tissues with digestion medium into 50 mL conical tubes. Mix very well by pipetting up and down.

9. Incubate the tubes in a 37 °C water bath with constant agitation at 75 rpm for 40–50 min. Check every 10 min to make sure the digestion is sufficient but not excessive (see Note 1).

10. Stop digestion by adding 10 mL basal medium to the digested tissue homogenate and pipetting to neutralize the collagenase.

11. Centrifuge at 500 × g for 5 min; remove the tubes and shake vigorously for 5 s, and centrifuge at 500 × g for 5 min.

12. Carefully aspirate the liquid layer and the primary mature adipocyte located on the top, and SVF appears as a brownish pellet on the bottom of the tube.

13. Add 10 mL basal medium to the pellet to resuspend the cells, and centrifuge at 500 × g for 5 min.

14. In the biosafety cabinet, carefully aspirate the medium without disturbing the pellet (see Note 2).

15. Resuspend the pellet in 10 mL basal medium, and filter the cell suspension into a new tube using a cell strainer (40 μm diameter).

16. Centrifuge at 500 × g for 5 min, and carefully aspirate the medium without disturbing the pellet.

### 3.2 SVF Monolayer Culture

1. Resuspend the pellet from Subheading 3.1, step 16, in 10 mL pre-warmed basal medium.

2. Plate the cells evenly onto a 10-cm collagen-coated plate, and place in a CO₂ incubator (37 °C, 5 % CO₂) (see Note 3).

3. Gently aspirate medium 2 h after cell plating, and wash the cells three times with PBS (see Note 4).

4. Add 10 mL fresh pre-warmed basal medium to each dish, and place back into the CO₂ incubator (37 °C, 5 % CO₂). Change medium every 2 days.
3.3 Induction of Adipogenesis

1. When the cell subcultures grow to a 95 % confluence for eWAT or 2 days after confluence for sWAT (day 0), replace the basal medium with differentiation medium. Supply fresh differentiation medium every 2 days.

2. Four days later (day 4), replace the differentiation medium with maintenance medium. Supply fresh maintenance medium every 2 days. Development of lipid droplets in the cells becomes obvious within 4 days after the addition of differentiation media.

3. Four days later (day 8), lipid accumulation is complete, and adipocytes can be harvested for protein analysis and Oil Red O staining (see Note 6).

3.4 Detection of Adipogenesis

1. Dissolve 0.5 g Oil Red O in 100 mL isopropanol, and filter through a 0.2-μm filter.

2. Immediately before staining experiments, add 30 mL of the Oil Red O stock to 20 mL distilled water to make Oil Red O working solution. Use this solution within 24 h of its preparation, and filter again if precipitation is present.

3. Remove maintenance medium and wash the cells three times with PBS.

4. Add 10 % buffered formalin (i.e., 4 % formaldehyde) sufficient to completely cover the cells and let sit for 10 min at room temperature.

5. Remove fixative, and rinse cells with PBS for 1 min, followed by two washes with water for 1 min.

6. Let the cell monolayers air dry for 10 min.

7. Stain the cells with Oil Red O solution (0.5 % in 60 % isopropanol) for 30 min, and rinse with water 3 times.

8. Visualize the stained cells on an inverted microscope system (Fig. 1).

9. Extract Oil Red O from the cells using 100 % isopropanol, gentle agitation for 15 min at room temperature.

10. Transfer the extracts to a 96-well plate (100 μl per well), and quantify lipid accumulation in the differentiated cells by measuring absorbance at 510 nm on a microplate reader (Fig. 1).

11. Additional detection of adipogenesis is performed by western blot analysis of PPARγ (a master adipogenic regulator) and adiponectin (an adipokine secreted by differentiated adipocytes).
using the standard procedure as described previously [1, 23], which shows upregulation of PPARγ and adiponectin (Fig. 2). In line with the pattern of lipid accumulation (Fig. 1), PPARγ and adiponectin were upregulated to a greater extent in SVF isolated from sWAT than from eWAT after differentiation induction.

4 Notes

1. If a 37 °C water bath is unavailable, the tissue digestion can be performed in an incubator shaker with setting at 150 rpm, 37 °C. Typically, a well-digested tissue appears to be a cloudy solution, with no chunks of fat remaining. Over-digestion of tissues will reduce cell yield and viability.

2. Sterile biosafety hood should be used in the following steps.

3. SVF isolated from 2–3 mice show good cell density on a 10-cm dish. A noncoated plate also works, but it takes a longer time (i.e., overnight incubation) for adipogenic cells to fully adhere to the plates. It would reduce adipogenic cell density if the first medium change takes place within 2 h after cell plating in step 3 in subheading 3.2.
4. This washing step is critical to remove contaminated red blood cells, immune cells, and other contaminants. Alternatively, ACK lysis buffer (154 mM NH₄Cl, 10 mM KHCO₃ and 0.1 mM EDTA, pH 7.4) can be used to remove red blood cells by resuspending the SVF pellet and incubating for 5 min at room temperature. After centrifuging at 500 × g for 5 min, remove the ACK buffer and wash SVF with basal medium once, then continue with step 15 in Subheading 3.1.

5. The primary stromal vascular cells grow much slower when they reach passage 3 or higher. We use cells at passage 2 for differentiation experiments. No difference is observed between collagen coated and non-coated plates during the cell subculture and adipogenic study.

6. SVF isolated from sWAT has a greater differentiation potential than that from eWAT, presumably because the latter requires a 3D structural support to stimulate its intrinsic differentiation potential. Indeed, SVF from eWAT pads exhibits robust differentiation in a 3D hydrogel system [24].
Acknowledgment

This work was supported in part by USDA National Institute of Food and Agriculture Hatch Project 1007334 (Z.C.).

References

Thermogenic Fat
Methods and Protocols
Wu, J. (Ed.)
2017, XI, 214 p. 42 illus., 30 illus. in color., Hardcover
ISBN: 978-1-4939-6819-0
A product of Humana Press