Chapter 7

Isolation and Fluorescence-Activated Cell Sorting of Murine WT1-Expressing Adipocyte Precursor Cells

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Abstract

The current global obesity epidemic has triggered increased interest in adipose tissue biology. A major area of attention for many is adipose tissue development. A greater understanding of adipocyte ontogeny could be highly beneficial in answering questions about obesity-associated disease. Recent work has shown that a proportion of mature adipocytes in visceral white adipose tissue are derived from Wt1-expressing adipocyte precursor cells. These adipocyte precursor cells reside within the adipose tissue itself, and are a constituent of the stromal vascular fraction (SVF), along with other, non-adipogenic, cell types. Crucially, heterogeneity exists within the adipocyte precursor population, with only a proportion of cells expressing Wt1. Moreover, it appears that this difference in the precursor cells may influence the mature adipocytes, with Wt1-lineage-positive adipocytes having fewer, larger lipid droplets than the Wt1-lineage negative. Using fluorescence-activated cell sorting, based on specific marker profiles, it is possible to isolate the adipocyte precursor cells from the SVF. Subsequently, this population can be divided into Wt1-expressing and non-expressing fractions, therefore permitting further analysis of the two cell populations, and the mature adipocytes derived from them. In this chapter we outline a method by which adipocyte precursor cells can be isolated, and how, using a specific mouse model, Wt1-expressing and non-expressing cells can be separated.

Key words Adipose tissue, Visceral, Adipocyte precursor, Adipocyte, Stromal vascular fraction, Fluorescence-activated cell sorting, Flow cytometry

1 Introduction

In recent years, interest in adipose tissue biology has substantially increased, driven by the obesity epidemic. Particular focus lies on the developmental origins of adipose tissue and the signaling mechanisms responsible for controlling its development and growth, two areas that remain relatively poorly understood [1].

1.1 Adipose Tissue Overview

In mammals, adipose tissue has traditionally been divided into two types: white adipose tissue (WAT) and brown adipose tissue (BAT). While both are implicated in energy balance, WAT is predominantly involved in the storage and mobilization of triglycerides, while BAT functions to burn fat and increase energy expenditure

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through adaptive thermogenesis [2]. Crucially, WAT has an exceptional capability for expansion, both through increasing adipocyte cell size (hypertrophy) and adipocyte number (hyperplasia), a key factor in the intensifying obesity crisis [3]. A third form of adipose tissue has recently been identified: "beige." "Beige" adipocytes are found within WAT, but display key characteristics of BAT, such as the expression of uncoupling protein-1 (UCP-1): essential for thermogenesis [4].

WAT is further divided into subcutaneous ("good fat") and visceral ("bad fat") depots. Visceral WAT is located within the body cavity, surrounding the major organs, while subcutaneous WAT resides under the skin. Visceral WAT is principally associated with metabolic syndrome and is a large risk factor for the development of cardiovascular disease, diabetes, and certain cancers, while subcutaneous WAT is thought to be protective [1, 5]. In this chapter, the focus is on visceral WAT. The majority of WAT mass can be accounted for by lipid-filled mature adipocytes; however, in terms of cell number, they make up less than half. The remaining cells comprise the stromal vascular fraction (SVF), and include blood, endothelial and mesenchymal cells. Importantly, adipocyte precursor cells reside within this SVF, and it is the presence and differentiation of these cells that determine adipocyte number, both during normal development and in obesity [6].

An important step within the field has been the development of a method by which adipocyte precursor cells can be separated from the non-adipogenic cells that comprise the rest of the SVF. Not only has this meant that it is now possible to isolate the adipocyte precursors, but it has also revealed the identity of the adipogenic SVF cells. Using multicolor flow cytometry, Rodeheffer et al. [7] isolated adipocyte progenitor cells with a specific cell surface marker profile (Lin–CD29+CD34+Sca1+CD24+), going on to show that these cells have the ability to differentiate into functioning WAT in vivo [7]. In addition to this, they went on to identify a pre-adipocyte population, with a slightly different marker profile (Lin–CD29+CD34+Sca1+CD24–) [8]. These cells can form adipocytes, but have a more limited adipogenic potential than the progenitors, and are unable to form fully functioning WAT depots in vivo. Additionally, these pre-adipocyte cells express adipogenic markers, suggesting that they are more committed to the adipocyte lineage than the progenitors [8]. In support of this, they also found that there is an increase in the latter (CD24–) and a decrease in the former (CD24+) populations postnatally, as the cells are further committed to becoming mature adipocytes [8]. Consequently, these cell surface marker profiles are now used to isolate adipocyte precursor cells by fluorescence-activated cell sorting (FACS).

1.2 Wt1 Expression in Adipose Tissue

Wt1 is expressed in the six murine visceral WAT depots: omental, epididymal, mesenteric, pericardial, retroperitoneal, and perirenal, but not in subcutaneous WAT or BAT [9]. Moreover, Wt1 is not

expressed in the mature adipocytes, but instead in the adipocyte precursor cells that reside in the SVF of the adult adipose depots [10]. Regarding the marker profiles described above, Chau et al. (2014) showed that 90% of the Wt1-expressing cells in the adult SVF fall into one of these two populations (Lin-CD31-CD29+CD34+), with the large majority (60–90% depending on depot) being in the CD24– pre-adipocyte category [10].

Based on the knowledge that Wt1 is expressed in visceral adipocyte precursor cells, lineage-tracing analysis has been used to investigate whether mature visceral fat arises from Wt1-expressing cells during embryonic and postnatal development [10]. Reporter *mTmG* mice were crossed with knock-in mice expressing tamoxifen-inducible Cre-recombinase at the *Wt1* locus (*Wt1CreERT2*; *mTmG*). With the *mTmG* reporter model, Tomato is ubiquitously expressed prior to Cre-mediated loxP recombination. Recombination leads to the removal of Tomato, causing membranous GFP to be expressed. Crucially, all progeny of the Cre-expressing cells are also labeled [11]. This lineage-tracing revealed that, depending on visceral depot, 28–77% of the mature adipocytes were GFP positive, thus indicating that they were derived from Wt1-expressing precursor cells [10]. In this particular study, tamoxifen was injected into the mother when the embryos were at E14.5, and the adult tissues analyzed at 1.2 years old. Henceforth, with this lineage tracing being reliant on a single dose of tamoxifen, it is likely that these percentages are an underestimate. Further work, involving tamoxifen dosing at 3 weeks old, has also demonstrated that a proportion of mature visceral adipocytes arise from cells expressing Wt1 postnatally [10]. Therefore we know that Wt1-expressing adipocyte precursor cells contribute to mature visceral but not subcutaneous WAT throughout development.

It is well known in the field that molecular differences exist between different WAT depots [2, 12]. In addition to this, with the knowledge that only a proportion of mature visceral adipocytes are derived from Wt1-expressing cells, we now know that within each of the WAT depots, heterogeneous populations of adipocyte precursor cells exist [10]. Moreover, work both in vivo and in an ex vivo culture system indicates that adipocytes derived from Wt1-positive cells have fewer, larger lipid droplets than those in the Wt1-negative lineage, while total lipid content and adipocyte cell size are the same [10], therefore implying that heterogeneity among the adipocyte precursors does influence the mature adipocyte and that Wt1 may have a role in this. In order to further explore the differences between the Wt1-expressing and non-expressing adipocyte precursors, as well as the mature adipocytes derived from these two lineages, it was important to develop a protocol by which these two adipocyte precursor populations could be effectively separated. For this we have utilized the Wt1-green fluorescent protein (GFP) knock-in mouse model, whereby Wt1-expressing cells are labeled with GFP [13].

2 Methods: Fluorescence-Activated Cell Sorting (FACS) Protocol for Isolating Murine Wt1-Expressing Adipocyte Precursor Cells

2.1 Introduction

This protocol was designed to permit the separation of Wt1-expressing (GFP+) and non-expressing (GFP–) adipocyte precursor cells from Wt1-GFP adult mouse visceral WAT depots. Isolated cells can subsequently be used for RNA extraction and gene expression analysis, as well as cell culture and in vitro differentiation. This protocol can either be used to simply separate all Wt1-positive and negative SVF cells, or to firstly isolate specific cells based on the marker profiles outlined by Rodeheffer et al. [7], before going on to separate these based on Wt1 (GFP) expression [7]. Presented here is a description of the tissue isolation, FACS sample preparation, and FACS analysis protocols.

2.2 Materials

1. Sterile dissection kit.
2. Carbon steel surgical blades.
3. 10 cm Culture dish.
4. Collagenase B (Roche).
5. Phosphate-buffered saline (PBS), pH 7.4.
6. Bovine serum albumin (BSA).
7. Penicillin/streptomycin (P/S).
8. 0.22 μM Millex syringe drive filter unit (Millipore).
9. 50 mL Syringe.
10. Rocking chamber heated to 37 °C.
11. 70 μm Nylon cell strainer (Corning).
12. Bench centrifuge with swinging bucket rotor.
13. Sterile cell culture plastic pipettes individually wrapped (5, 10, 25 mL).
14. Pipette aid.
15. Sterile conical centrifuge tubes (15 and 50 mL).
16. Cell aspirator.
17. Fetal calf serum (FCS).
18. FACS buffer: PBS, 5% (v/v) FCS, 1% (v/v) P/S.
19. 5 mL Polystyrene round-bottom tube with cell strainer cap (35 μm) (Corning).
20. Biotin mouse lineage panel (containing lineage markers: CD3e, CD11b, CD45R (B220), Gr-1, Ly-76) (BD Biosciences).
21. Antibodies (*see* Table 1).
22. OneComp eBeads (eBioscience).
23. Culture medium: Dulbecco's modified Eagle medium 1× (DMEM), 10% (v/v) fetal calf serum, 1% (v/v) penicillin/streptomycin, 0.5% (v/v) sodium pyruvate (if culturing sorted cells).

Table 1
Markers and fluorochromes used in the flow cytometry panel for the isolation of adipocyte precursors, including dilutions and excitation/emission wavelengths [6]

Antibody	Gene name	Supplier	Fluorochrome	Excitation laser (nm)	Emission (nm)	Dilution[a]
CD31	Platelet/endothelial cell adhesion molecule 1	eBioscience	PerCP-eF710	488 (Blue)	710	1:100
CD29	Integrin β1	eBioscience	APC	637–647 (Red)	660	1:100
CD24	Heat-stable antigen	eBioscience	eF780	633–647 (Red)	780	1:200
CD34	Cluster differentiation hematopoietic progenitor cell antigen	Biolegend	PE-Cy5	488 (Blue)	670	1:50
Sca-1	Stem cell antigen-1	Biolegend	Pacific Blue	405 (Violet)	455	1:160
Streptavidin[b]		eBioscience	PerCP-Cy5.5	488 (Blue)	690	1:340

[a]Dilutions are a recommendation based on our work. The user should determine the optimal dilution for different antibodies, fluorochromes, and FACS machines

[b]Secondary antibody to detect the biotinylated primary antibodies of the mouse lineage panel

24. TRIzol® Reagent (Invitrogen) (if extracting RNA from sorted cells).

2.3 Methods

2.3.1 Isolation of Stromal Vascular Fraction Cells

1. Euthanize mice by cervical dislocation and dissect out required adipose depots.
2. Dissolve collagenase B at a concentration of 1 mg/mL in sterile PBS containing 4 mg/mL bovine serum albumin and 1% (v/v) penicillin/streptomycin (*see* **Note 1**).
3. In a 10 cm culture dish, using carbon steel surgical blades, chop the fat pads into 1–2 mm pieces (*see* **Note 2**).
4. Transfer the chopped fat tissue and 5–10 mL collagenase B solution into a 15 mL tube and incubate at 37 °C for 1 h in a rocking chamber.
5. Shake the samples vigorously by hand to obtain a single-cell suspension.
6. Filter the suspension through a 70 μm nylon cell strainer into a 50 mL Falcon tube.
7. Centrifuge the tube at 300 × *g* for 5 min at 4 °C in a swinging bucket rotor.

8. Using vacuum aspiration, remove the supernatant containing the enzyme and mature adipocytes (*see* **Note 3**).
9. Resuspend the stromal vascular fraction (SVF) cell pellet in 1 mL FACS buffer.

2.3.2 FACS Sample Preparation/Antibody Staining

1. If sorting based on cell surface marker profiles, dilute required antibodies to appropriate concentrations in FACS buffer (Table 1; suggested antibody concentrations). Follow the manufacturer's instructions for diluting antibodies from the biotin mouse lineage panel (*see* **Note 4**).
2. Filter the SVF suspension (from Subheading 2.1.9) into a 5 mL polystyrene round-bottom tube with cell strainer cap (35 μm) (*see* **Note 5**).
3. Centrifuge the tube at 300 × *g* for 5 min at 4 °C in a swinging bucket rotor (*see* **Note 6**).
4. Tip off the supernatant and resuspend the pellet in the appropriate antibody staining solution (~100 μL). Incubate the suspension in the dark at room temperature for 15 min (*see* **Note 7**). If simply sorting all Wt1-positive and Wt1-negative cells, so NOT antibody staining, pellet can be resuspended in ~150 μL FACS buffer, and go directly to **step 7**.
5. To wash, add 3 mL FACS buffer to each sample and centrifuge at 300 × *g* for 5 min at 4 °C. Tip off the supernatant and repeat the staining procedure for secondary/additional antibodies if required.
6. Once staining is complete, resuspend the final pellet in ~150 μL FACS buffer. The cells are now ready for FACS.
7. FACS-separated cells will be collected into either 1.5 mL Eppendorf tubes or 15 mL Falcon tubes. If sorted cells are to be cultured in vitro, these tubes should be filled with culture media prior to sorting. Alternatively, if RNA is to be extracted from the sorted cells, they can be sorted directly into 500 μL TRIzol® Reagent.

2.3.3 FACS/Flow Cytometry

1. We use a BD FACSAria II laser sorter/analyzer to sort and analyze our SVF samples along with BD FACSDiva and FlowJo software.
2. Compensation: When performing multicolor flow cytometric analysis (as described here), it is important that any potential overlap between the emission spectra of two or more fluorochromes is eliminated. This overlap can occur when more than one detector is capable of measuring a particular fluorescent dye. If this step is not carried out correctly it may be the case that a population of cells negative for an antigen of interest will appear positive, due to spillover of the fluorescent signal from another fluorescent antibody with a similar emission spectra. This compensation can be carried out using the single-color compensation controls (antibody-capture beads and SVF cells) outlined in Table 2. Each bead/cell sample is stained with just

Table 2 Proposed sample setup for the isolation of adipocyte precursors [14]

Single-color compensation controls (capture beads/cells)	Lin (biotin mouse lineage panel)	Streptavidin PerCP-Cy5.5	CD31 PerCP-eF710	CD29 APC	CD24 eF780	CD34 PE-Cy5	Sca-1 Pacific Blue
Beads—unstained	–	–	–	–	–	–	–
Beads—Lin-SA.PerCP-Cy5.5	+	+	–	–	–	–	–
Beads—CD31-PerCPeF710	–	–	+	–	–	–	–
Beads—CD29-APC	–	–	–	+	–	–	–
Beads—CD24-eF780	–	–	–	–	+	–	–
Beads—CD34-PECy5	–	–	–	–	–	+	–
Beads—Sca1-PB	–	–	–	–	–	–	+
SVF cells—unstained	–	–	–	–	–	–	–
SVF cells—Lin-SA.PerCP-Cy5.5	+	+	–	–	–	–	–
SVF cells—SA.PerCP-Cy5.5	–	+	–	–	–	–	–
SVF cells—CD31-PerCPeF710	–	–	+	–	–	–	–
SVF cells—CD29-APC	–	–	–	+	–	–	–
SVF cells—CD24-eF780	–	–	–	–	+	–	–
SVF cells—CD34-PECy5	–	–	–	–	–	+	–
SVF cells—Sca1-PB	–	–	–	–	–	–	+
Fluorescence-minus-one controls							
SVF cells—Lin-SA.PerCP-Cy5.5	–	–	+	+	+	+	+
SVF cells—CD31-PerCPeF710	+	+	–	+	+	+	+
SVF cells—CD29-APC	+	+	+	–	+	+	+
SVF cells—CD24-eF780	+	+	+	+	–	+	+
SVF cells—CD34-PECy5	+	+	+	+	+	–	+
SVF cells—Sca1-PB	+	+	+	+	+	+	–
Test samples: SVF cells (all markers)	+	+	+	+	+	+	+

one of the fluorescent antibodies from the multicolor panel. The beads/cells are then gated on to gain the single-color controls for each dye [6, 14].

3. Fluorescence-minus-one (FMO) controls: To support the compensation controls, and ensure that any observed fluorescent signal has arisen from the antibody of interest only, FMO controls should be carried out. For each fluorescent antibody used, a sample of SVF cells should be stained with all other antibodies in the panel, excluding the one that has been compensated for. This FMO control and the experimental sample (stained with the entire panel of antibodies) should now be analyzed using the same compensation settings (those set up for the antibody being verified). If the compensation settings are correct, a signal should be observed for the antigen of interest in the experimental sample only, and not in the FMO control. If a fluorescent signal is observed in the FMO control, this is the result of a signal from another antibody spilling over, and the compensation settings should be adjusted in order to eliminate this [6]. *See* Table 2 for the suggested FMO control setup.
4. Regarding data acquisition, typically we begin by generating a forward scatter height (FSC-H) vs. forward scatter area (FSC-A) plot to select for singlets and remove doublets. Subsequently, this population of cells is gated on side scatter area (SSC-A) vs. FSC-A to remove debris. This main population of cells is then gated further, including all the fluorescent parameters, in order to obtain the required cell populations (Fig. 1a). When sorting into Wt1+ (GFP+) and Wt1− (GFP−) fractions, it is important that the GFP gate is established on a sample of SVF cells negative for GFP (Fig. 1b).

3 Notes

1. Allow for 5–10 mL of collagenase solution for digestion of one adipose depot. If sorted cells are to be cultured, collagenase solution should be sterilized before use, by filtration through a syringe-driven filter unit (0.22 μM).
2. If sorted cells are to be cultured, all procedures from and including **step 3** must be carried out in a sterile culture hood (where possible), using sterile conditions.
3. The cells of the SVF will pellet at the bottom of the Falcon tube, while the mature adipocytes will float to the top of the supernatant.
4. For all antibodies listed in Table 1, approximately 100 μL of staining solution is sufficient to stain the SVF from one adipose depot, although exact volumes should be determined by the individual user.

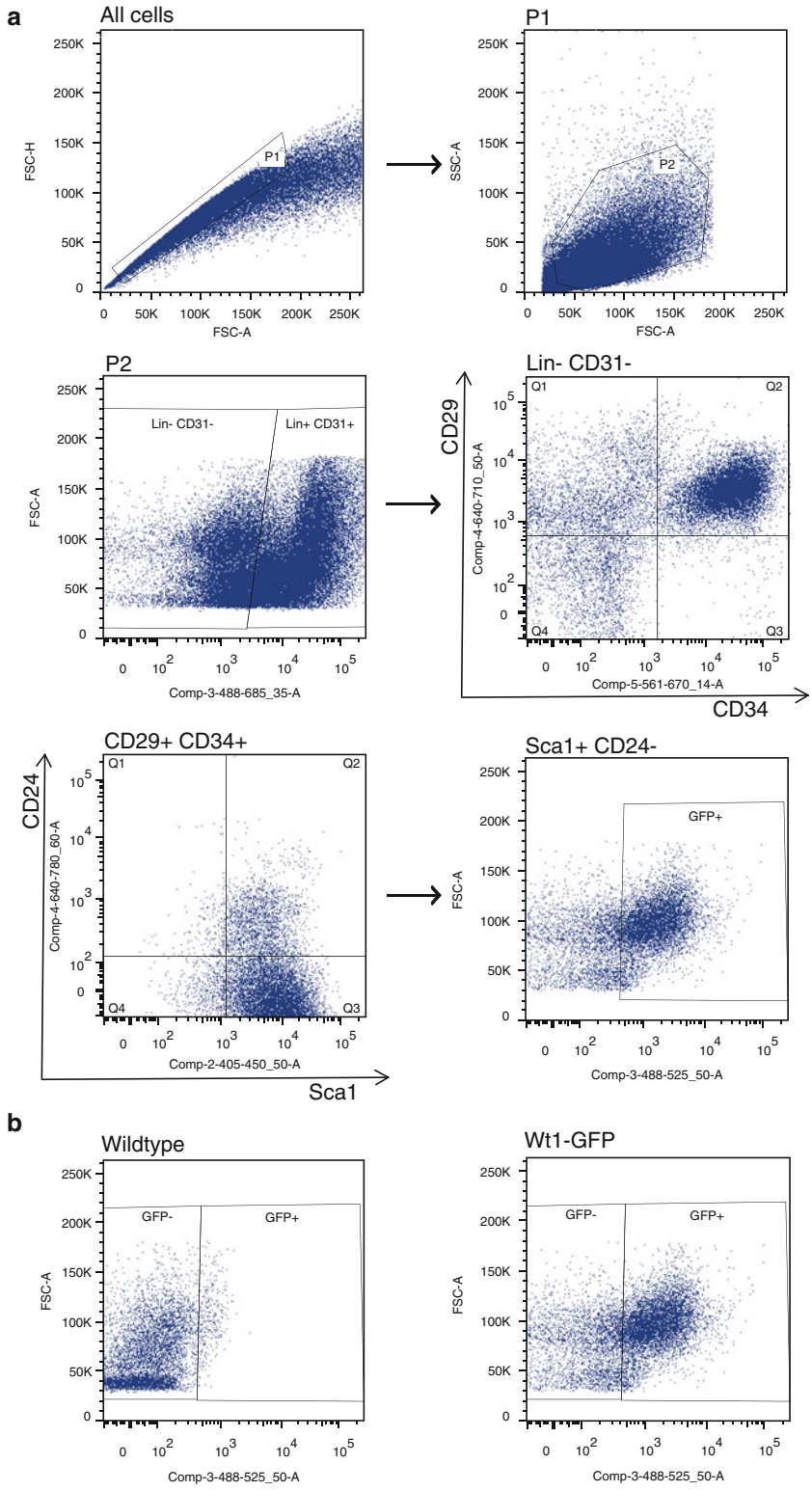

Fig. 1 Example flow cytometry gating for the isolation of Wt1+ (GFP+) Lin− CD31− CD29+ CD34+ Sca1+ CD24− pre-adipocytes from the stromal vascular fraction (**a**). A non-GFP-expressing cell sample should be used to establish the GFP+ gate (**b**)

5. If sorting cells based on a specific marker profile, compensation and fluorescence-minus-one controls must also be included. For each marker used, filter 2× 100 μL of the cell suspension into two separate 5 mL polystyrene round-bottom tubes with cell strainer caps (35 μm). One of these samples should be incubated with one antibody only (single-color compensation sample), and the other should be incubated with all antibodies being used, except the one being compensated for (fluorescence-minus-one sample), as outlined in Table 2. Additionally, for each marker used, 7 μL of OneComp eBeads should be incubated with an individual antibody, and treated as one of the compensation samples.
6. If cells are to be used for culture and must remain in sterile conditions, the FACS tube cell strainer cap should be replaced with a snap cap prior to centrifugation.
7. If sorting cells based on a specific marker profile (i.e.: Lin-CD29+CD34+Sca1+CD24−), primary antibodies from the biotin mouse lineage panel should be added to the cells first (made up into one solution according to the manufacturer's protocol). Unlike the other antibodies (Table 1), those in the lineage panel are not conjugated to a fluorophore, but are biotinylated, so a secondary streptavidin fluorochrome conjugate must be added in a second incubation (streptavidin PerCP-Cy5.5). Following this, all other required antibodies (Table 1) can be added in a single step.

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