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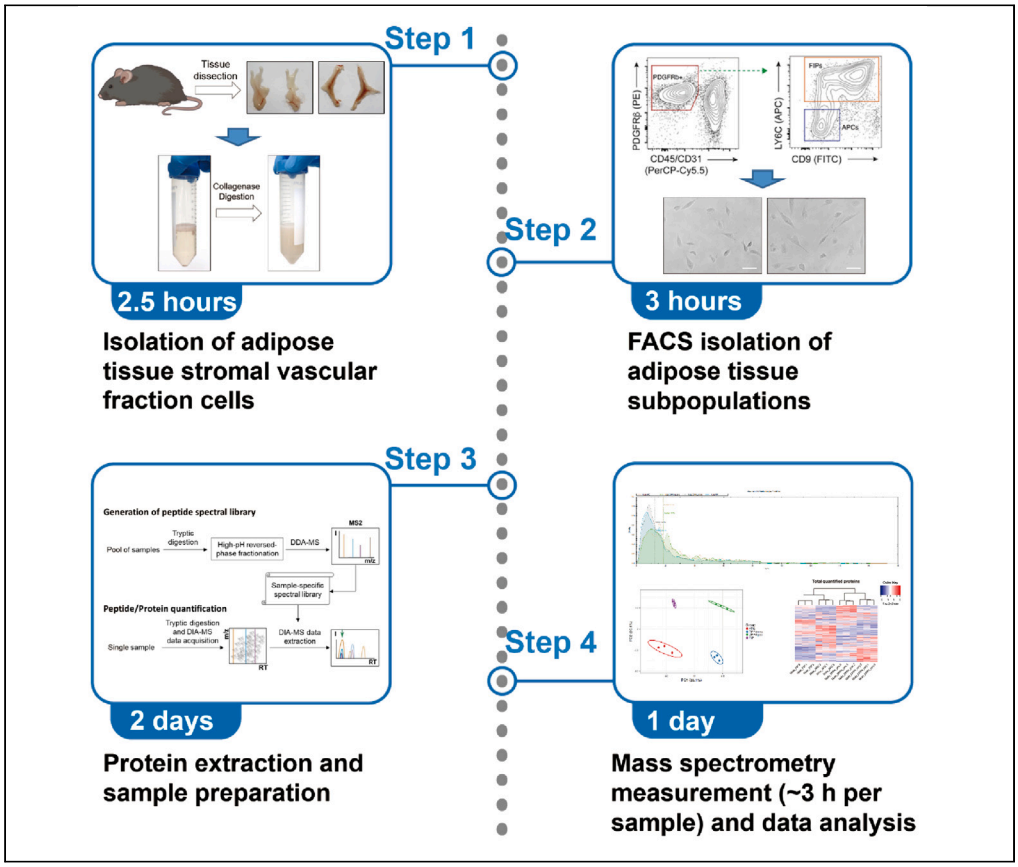
Protocol for quantitative proteomic analysis of heterogeneous adipose tissue-residing progenitor subpopulations in mice



Recent studies have revealed cellular heterogeneity of mesenchymal stromal cells and immune cells in adipose tissue and emphasized the need for quantitative analysis of small numbers of functionally distinct cells using state-of-the-art "omics" technologies. Here, we present an optimized protocol for precise protein quantification from minute amounts of samples. We describe steps for isolation of mouse adipose progenitor cells, proteomics sample preparation, mass spectrometry measurement, and computational analysis. This protocol can be adapted to other samples with limited amounts.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

Bo Shan, Clive S. Barker, Harry Theraulaz, ..., Rana K. Gupta, Mengle Shao, Yibo Wu

boshan@zju.edu.cn (B.S.)
mlshao@ips.ac.cn (M.S.)
wulabproteomics@gmail.com (Y.W.)

Highlights

Optimized protocol for isolation of mouse adipose progenitor cells

Proteomics sample preparation from limited amount of material

Sensitive and precise protein quantification using DIA-MS and computational analysis

Applicable to a wide range of samples with small amounts

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Protocol for quantitative proteomic analysis of heterogeneous adipose tissue-residing progenitor subpopulations in mice

Bo Shan,[1,2,7,*] Clive S. Barker,[3,7] Harry Theraulaz,[4] Xiaoli Zhang,[5] Yan Ping,[1,2] Rana K. Gupta,[6] Mengle Shao,[5,*] and Yibo Wu[3,4,8,*]

[1]Zhejiang Provincial Key Laboratory of Pancreatic Disease, The First Affiliated Hospital, Institute of Translational Medicine, Zhejiang University School of Medicine, Zhejiang University, Hangzhou, China

[2]Cancer Center, Zhejiang University, Hangzhou, China

[3]YCI Laboratory for Next-Generation Proteomics, RIKEN Center of Integrative Medical Sciences, 1-7-22 Suehiro-cho, Tsurumi-ku, Yokohama, Kanagawa 230-0045, Japan

[4]Chemical Biology Mass Spectrometry (ChemBioMS) Platform, Faculty of Science, University of Geneva, 1211 Geneva, Switzerland

[5]CAS Key Laboratory of Molecular Virology and Immunology, The Center for Microbes, Development and Health, Shanghai Institute of Immunity and Infection, Chinese Academy of Sciences, Shanghai, China

[6]Department of Medicine, Division of Endocrinology, Duke Molecular Physiology Institute, Duke University, Durham, NC, USA

[7]Technical contact

[8]Lead contact

*Correspondence: boshan@zju.edu.cn (B.S.), mlshao@ips.ac.cn (M.S.), wulabproteomics@gmail.com (Y.W.)
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SUMMARY

Recent studies have revealed cellular heterogeneity of mesenchymal stromal cells and immune cells in adipose tissue and emphasized the need for quantitative analysis of small numbers of functionally distinct cells using state-of-the-art "omics" technologies. Here, we present an optimized protocol for precise protein quantification from minute amounts of samples. We describe steps for isolation of mouse adipose progenitor cells, proteomics sample preparation, mass spectrometry measurement, and computational analysis. This protocol can be adapted to other samples with limited amounts.

For complete details on the use and execution of this protocol, please refer to Shan et al. (2022).[1]

BEFORE YOU BEGIN

Institutional permissions

Animal experiments should adhere to ethical guidelines and principles approved by an institutional animal care and use committee. The use of animals in this study followed the procedures approved by the ethics committee of RIKEN.

Experimental concerns

Recent progress in single-cell/nucleus RNA sequencing has uncovered extensive cellular heterogeneity of both mouse and human white adipose tissue (WAT) mesenchymal stromal cells which contain adipose progenitor subpopulations.[2–13] These studies have independently confirmed the existence of heterogeneous stromal cells within adipose depots, which can differ across depot and between sex.[1,14] In the inguinal and gonadal adipose depots of adult mice, a minimum of two distinct subpopulations of mesenchymal stromal cells have been identified.[14] One subpopulation enriched in the expression of adipocyte markers (e.g., *Pparg*, *Cd36*, and *Lpl*) represents

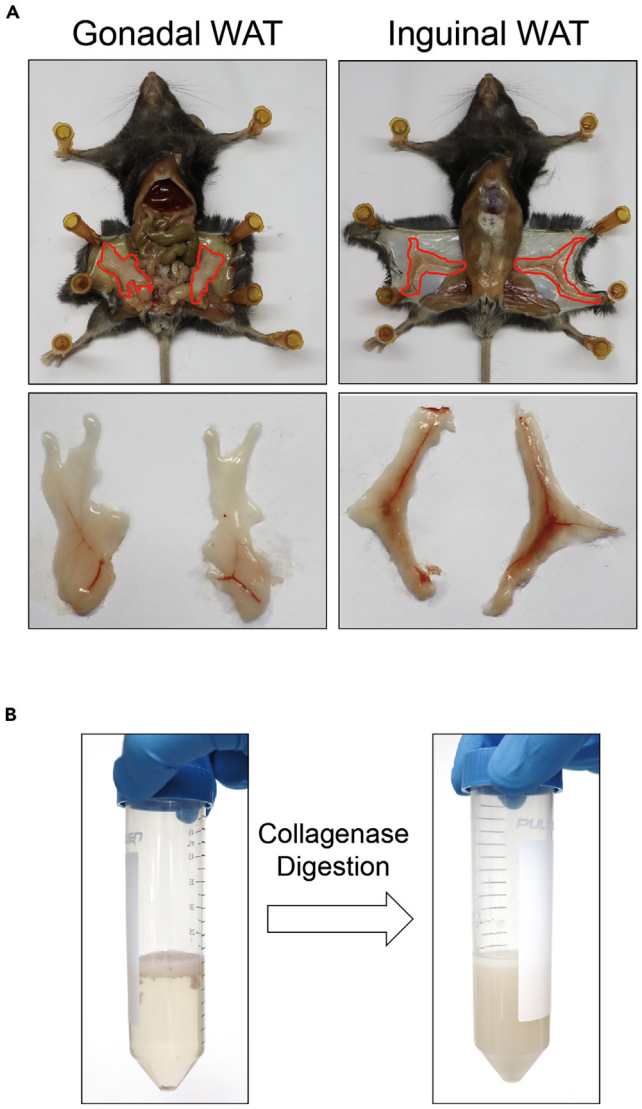

Figure 1. Dissection and digestion of inguinal and gonadal WAT depots for the isolation of adipose progenitor populations

(A) Anatomical positions of inguinal and gonadal WAT depots in a 8-week old male C57BL/6 mouse.

(B) Photographs of isolated WAT tissue before and after collagenase digestion.

"committed preadipocytes" with high adipogenic potential. Another major mesenchymal stromal cell subpopulation, often characterized by *Dpp4* expression, lacks the expression of adipogenic markers, thereby suggesting a possible classification of "early preadipocytes"[6] or non-adipogenic cells.[5,10]

This protocol is based on the isolation and analysis of these two major adipocyte progenitor subpopulations within inguinal WAT and gonadal WAT in male C57BL/6 mice, as previously published in the original studies.[1,5,13] Considering murine adipocyte progenitor subpopulations are heterogeneous across depots and sexes,[1,4,7,13] the procedures applied for adipocyte progenitor isolation from other WAT depots or from female mice require additional modifications.

The experimentalist needs to be familiar with the anatomy of inguinal and gonadal WAT depots. The anatomical localizations of these two depots are shown in (Figure 1A). The following protocol has

been established using 6–8-week-old wild-type male mice with C57BL/6 genetic background and 20–25 *g* body weight.

Preparation of buffers

Note: Commercially available 1X ready-to-use solutions including PBS, DPBS and HBSS are stored at room temperature according to the manufacturer's instructions. RBC lysis buffer is provided at a 10X concentration and needs to be diluted into 1X solution with ddH_2O before use.

Digestion buffer		
	Final concentration	Amount
HBSS	1 x	10 mL
Collagenase D	1 mg/mL	10 mg
BSA	15 mg/mL	150 mg
Total		**10 mL**

Flow buffer		
	Final concentration	Amount
DPBS	1 x	490 mL
FBS	2% (vol/vol)	10 mL
Total		**500 mL**

Note: Digestion buffer and flow buffer need to be freshly prepared at 1X concentration for each individual experiment.

Phase-transfer surfactant (PTS) protein extraction buffer		
Reagent	Final concentration	Amount
120 mM Sodium deoxycholate	12 mM	1 mL
120 mM Sodium *N*-Dodecanoylsarcosinate	12 mM	1 mL
Trizma Hydrochloride Solution, pH 9.0, 1 M Trizma base	100 mM	1 mL
Water, LC/MS Grade		7 mL
Roche cOmplete, mini, EDTA-free protease inhibitor cocktail		1 Tablet
Total		10 mL

Note: 1X PTS protein extraction buffer is made fresh and used within 24 hours, to ensure stability of the protease inhibitor cocktail. Stocks of 120 mM sodium deoxycholate and 120 mM sodium *N*-dodecanoylsarcosinate can be kept frozen for several months at −20°C.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
PerCP/Cyanine5.5 anti-mouse CD45 antibody (1:400 dilution)	BioLegend	Cat# 103132 RRID: AB_893340
PerCP/Cyanine5.5 anti-mouse CD31 antibody (1:400 dilution)	BioLegend	Cat# 102420 RRID: AB_10613644
PE anti-mouse CD140b (PDGFRβ) antibody (1:100 dilution)	BioLegend	Cat# 136006 RRID: AB_1953271
APC anti-mouse LY6C antibody (1:400 dilution)	BioLegend	Cat# 128016 RRID: AB_1732076
FITC anti-mouse CD9 antibody (1:200 dilution)	BioLegend	Cat# 124808 RRID: AB_1279321

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
BV421 anti-mouse CD26 (DPP4) antibody (1:200 dilution)	BD Biosciences	Cat# 740021 RRID: AB_2739793
Chemicals, peptides, and recombinant proteins		
Collagenase D	Roche	Cat# 11088882001
RBC lysis buffer	eBioscience	Cat# 00-4300-54
Phosphate-buffered saline (PBS)	Fisher Scientific	Cat# 21040CV
Dulbecco's phosphate-buffered saline (DPBS)	Sigma-Aldrich	Cat# D8537-500milliliter
Hank's balanced salt solution (HBSS)	Sigma-Aldrich	Cat# H8264
Bovine serum albumin (BSA)	Fisher Scientific	Cat# BP1605-100
Fc block (mouse CD16/CD32)	eBioscience	Cat# 553141
Fetal bovine serum (FBS)	Sigma-Aldrich	Cat# 12303C
Ammonium hydrogencarbonate (ammonium bicarbonate)	FUJIFILM Wako Pure Chemical Corporation	Cat# 018-21742; CAS RN: 1066-33-7
cOmplete tablets, mini EDTA-free, EASYpack protease inhibitor cocktail	Roche	Cat# 04 693 159 001
L-cysteine	FUJIFILM Wako Pure Chemical Corporation	Cat# 039-20652; CAS RN: 52-90-4
DL-dithiothreitol, ≥98% (HPLC), ≥99.0% (titration)	Sigma	Cat# D0632-1gram; CAS RN: 3483-12-3
Ethyl acetate	FUJIFILM Wako Pure Chemical Corporation	Cat# 057-03371; CAS RN: 141-78-6
Iodoacetamide	Sigma	Cat# I6125-25G; CAS RN: 144-48-9
Lysyl endopeptidase mass spectrometry grade (Lys-C)	FUJIFILM Wako Pure Chemical Corporation	Cat# 125-05061
Sodium deoxycholate	FUJIFILM Wako Pure Chemical Corporation	Cat# 190-08313; CAS RN: 302-95-4
Sodium N-dodecanoylsarcosinate (sodium *N*-lauroyl sarcosinate)	FUJIFILM Wako Pure Chemical Corporation	Cat# 192-10382; CAS RN: 137-16-6
Trifluoroacetic acid Optima LC/MS	Thermo Fisher Scientific	Cat# A116-50; CAS RN: 76-05-1
Sequencing grade modified trypsin	Promega	Cat# V5113
Trizma hydrochloride solution, pH 9.0, 1 M Trizma base	Sigma	Cat# T2819-100ML; CAS RN: 77-86-1
ZipTip C18 pipette tips with 0.6 µL C18 resin	Millipore	Cat# ZTC18S096
Water, Optima LC/MS	Thermo Fisher Scientific	Cat# W6-500; CAS RN: 7732-18-5
0.1% FA, Optima LC/MS	Thermo Fisher Scientific	Cat# LS118-500; CAS RN: 64-18-6, 7732-18-5
0.1% FA in acetonitrile, Optima LC/MS	Thermo Fisher Scientific	Cat# LS120-500; CAS RN: 64-18-6, 75-05-8
0.1% FA in 80% acetonitrile, Optima LC/MS	Thermo Fisher Scientific	Cat# LS122-500; CAS RN: 64-18-6, 75-05-8, 7732-18-5
0.1% TFA, Optima LC/MS	Thermo Fisher Scientific	Cat# LS119-500; CAS RN: 76-05-01, 7732-18-5
0.1% TFA in acetonitrile, Optima LC/MS	Thermo Fisher Scientific	Cat# LS121-500; CAS RN: 76-05-01, 75-05-8
Pierce BCA Protein Assay Kit	Thermo Fisher Scientific	Cat# 23227
Pierce High pH Reversed-Phase Peptide Fractionation Kit	Thermo Fisher Scientific	Cat# 84868
Pierce Quantitative Colorimetric Peptide Assay Kit	Thermo Fisher Scientific	Cat# 23275
Software and algorithms		
GraphPad Prism version 8.0	GraphPad Software, Inc	https://www.graphpad.com
FlowJo version 10.6.1	FlowJo, LLC	https://www.flowjo.com; RRID: SCR_008520
QIAGEN Ingenuity Pathway Analysis (QIAGEN IPA)	QIAGEN	https://digitalinsights.qiagen.com/products-overview/discovery-insights-portfolio/analysis-and-visualization/qiagen-ipa/
ClustVis	Metsalu and Vilo, 2015[23]	https://biit.cs.ut.ee/clustvis/
Proteome Discoverer 2.4	Thermo Fisher Scientific	https://www.thermofisher.com/jp/en/home/industrial/mass-spectrometry/liquid-chromatography-mass-spectrometry-lc-ms/lc-ms-software/multi-omics-data-analysis/proteome-discoverer-software.html
Spectronaut	Biognosys AG	https://biognosys.com/shop/spectronaut
R version 4.1.2	N/A	https://cran.r-project.org/bin/windows/base/
Other		
Spring scissors	Fisher Scientific	Cat# 08-953-1B
Centrifuge 5804 R	Eppendorf	Cat# 022629981

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
40 μm cell strainers	Fisher Scientific	Cat# 352340
100 μm cell strainers	Fisher Scientific	Cat# 352360
5 mL polypropylene tubes	Fisher Scientific	Cat# 352053
BD FACSAria III cell sorter	BD	N/A
Q Exactive Plus Orbitrap LC-MS/MS system, including a Nanospray Flex ion source and an EASY-nLC 1200 liquid chromatography apparatus	Thermo Fisher Scientific	N/A
Acclaim PepMap 100 C18 LC columns	Thermo Fisher Scientific	Cat# 164946
Analytical columns with 3-μm C18 particles, 75-μm inner diameter, and 12-cm filling length	Nikkyo Technos, Co. Ltd., Japan	Cat# NTCC-360/75-3-125
Centrifugal vacuum concentrator, miVac Duo concentrator/Duo pump	Genevac, UK	N/A
NanoDrop	Thermo Fisher Scientific	N/A
Benchtop centrifuge, Centrifuge 5427 R	Eppendorf, Germany	N/A
Sonicator, Bioruptor water bath	Cosmo Bio Co. Ltd., Japan	N/A
ThermoMixer C	Eppendorf	N/A

STEP-BY-STEP METHOD DETAILS

Isolation of stromal vascular fraction cells from gonadal and inguinal white adipose tissue

Timing: 2.5 h

1. Dissect gonadal and inguinal WAT from 6–8-week-old male mice and place isolated fat depots in ice-cold 1x DPBS solution.

   Note: Dissected WAT depots can be placed in the cold DPBS for a few hours prior to the following steps.

2. Combine isolated fat depots (2–4 depots from 1–2 mice recommended) and mince the tissue in a 10 mL beaker containing 200 μL digestion buffer using spring scissors.

   Note: The depots should be minced completely and chopped into pieces less than 1 mm diameter.

3. Transfer the minced tissue to a new 50 mL centrifuge tube containing 10 mL digestion buffer.
4. Incubate at 37°C in a water bath for around 1 h (gonadal WAT) or 1.5 h (inguinal WAT) with shaking (100 rpm).

   Note: Vortex the tube gently every 15 min during the digestion. WAT tissue is sufficiently digested when there is no apparent tissue debris and the digested tissue reaches a "soup-like" consistency (Figure 1B).

5. After incubation, filter the digested tissue through a 100 μm cell strainer to remove undigested tissue.

   Note: Well-digested tissue samples contain little residual material on the filters.

6. Dilute filtered samples to 30 mL with flow buffer, and centrifuge at 600 × *g* for 5 min at 4°C.
7. Aspirate the supernatant which contains floating mature adipocytes and keep the pellet which contains SVF cells.
8. Proceed immediately to Fluorescence Activated Cell Sorting (FACS) steps.

Isolation of adipose PDGFRβ+ subpopulations using FACS

Timing: 3 h

9. Dissolve the pellet in 1 mL of 1x RBC lysis buffer by shaking the tube gently.
10. Incubate at room temperature (RT) for 1 min.

Note: Prolonged incubation may reduce the viability of the cells.

11. Add 10 mL of flow buffer to stop the lysis and filter the cell mixture through a 40 μm cell strainer into another new 50 mL centrifuge tube.
12. Centrifuge the cell mixture at 600 × *g* for 5 min at 4°C.
13. Aspirate supernatant and resuspend the pellet in flow buffer. Adjust the cell concentration to around 10^6 cells per mL (400–800 μL per sample in total).
14. Add Fc block (1:200) Incubate at 4°C for 10 min. Centrifuge the cell mixture at 600 × *g* for 5 min at 4°C.
15. Resuspend the pellet by gentle pipetting in the flow buffer and transfer 400–800 μL a new 1.5 mL tube for the further flow antibody incubation as follows.
    a. Prepare control tubes for i, unstained cells, ii, single-color controls, and iii, fluorescence minus one (FMO) controls, respectively.
    b. For gonadal WAT SVF cells staining, the cells were incubated with the following flow antibodies and dilutions: anti-CD45 (1:400), anti-CD31 (1:400), anti-CD140b (PDGFRβ) (1:100), anti-LY6C (1:400), anti-CD9 (1:200).
    c. For inguinal WAT SVF cell staining, the cells were incubated with the following flow antibodies and dilutions: anti-CD45 (1:400), anti-CD31 (1:400), anti-CD140b (PDGFRβ) (1:100) and anti-DPP4 (1:200).
16. Incubate at 4°C for 15 min and all the tubes should be protected from light.

Optional: It is advisable that the tubes are placed on a shaking platform or shaken by hand every 5 min during the incubation.

17. Pellet by centrifugation at 600 × *g* for 5 min at 4°C.
18. Aspirate buffer and resuspend cells in 400 μL of flow buffer.
19. Pellet by centrifugation at 600 × *g* for 5 min at 4°C.
20. Aspirate buffer and resuspend pellets in 400 μL of flow buffer.
21. Pass through 40 μm filter caps into 5 mL round bottom polystyrene FACS tubes.
22. Take the following steps for FACS gating strategies.
    a. Use unstained and single-color controls for fluorescence compensation.
    b. Use FMO controls to set experimental gates.
    c. Use the gating strategy shown in Figure 2A to obtain adipocyte precursor cells (APCs) and fibro-inflammatory progenitors (FIPs) within gonadal WAT SVF cells.[5] Gate APCs as CD45- CD31- CD140b+ LY6C- CD9- and FIPs as CD45- CD31- CD140b+ LY6C + cells.

Note: CD45 is a common marker for hematopoietic immune cells and CD31 is the marker for endothelial cells. CD140b is used to label the PDGFRβ+ cells. Therefore, FIPs were sorted from CD45-CD31-PDGFRRβ+ cells with additional maker LY6C+ and APCs were defined as CD45-CD31-PDGFRRβ+LY6C-CD9-. Details for subpopulation gating are described in Figure 2A.

    d. Use the gating strategy shown in Figure 2B to obtain DPP4+ and DPP4- APCs within inguinal WAT SVF cells.[13,15] Gate DPP4+ APCs as CD45- CD31- CD140b+ DPP4+ and DPP4- APCs as CD45- CD31- CD140b+ DPP4-.

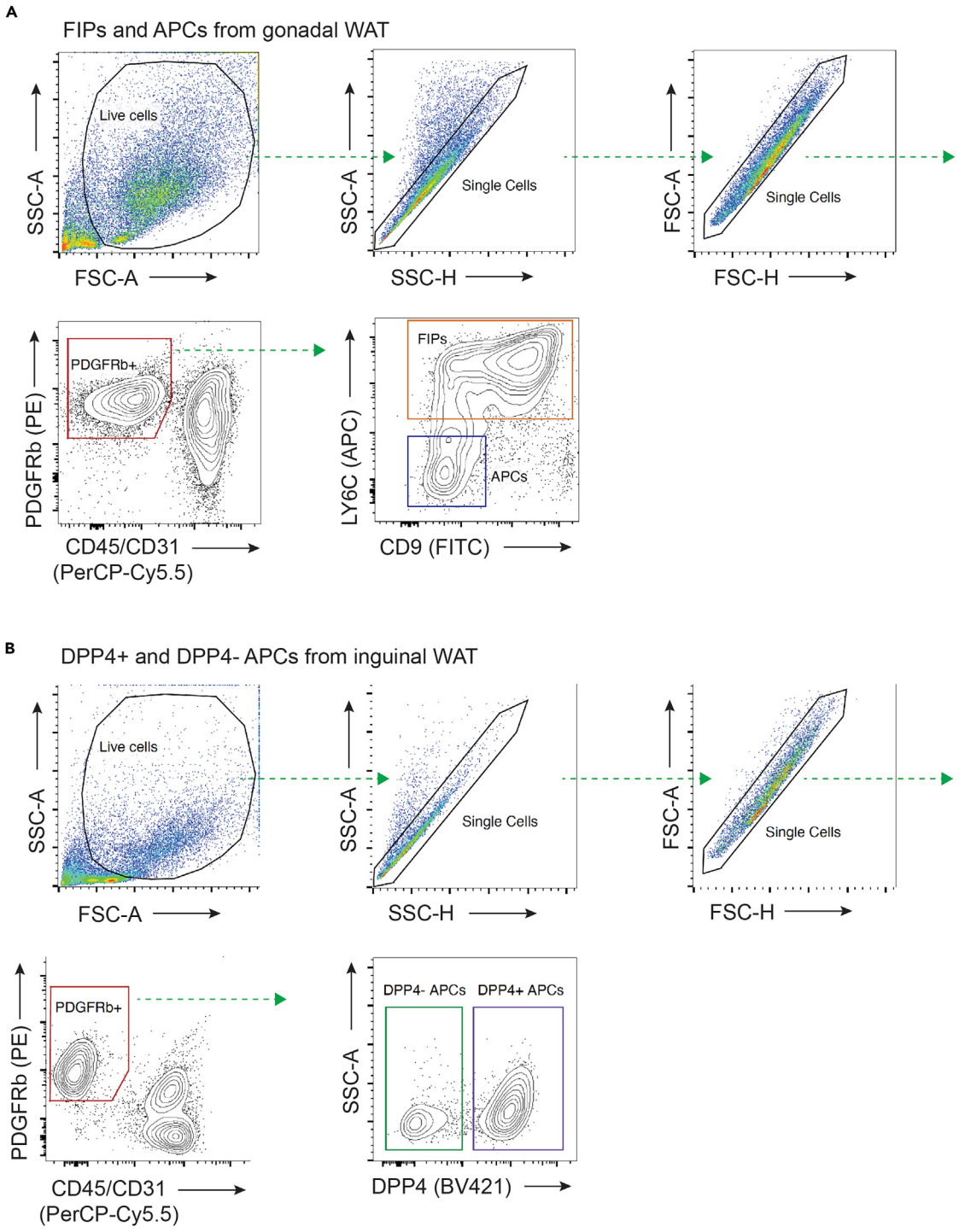

Figure 2. Isolation of PDGFRβ+ stromal cell subpopulations from gonadal and inguinal white adipose tissue by FACS

(A) Representative FACS collection gates for isolating FIPs and APCs from SVFs of gonadal WAT.

(B) Representative FACS collection gates for isolating DPP4+ and DPP4- APCs from SVFs of inguinal WAT.

Note: CD45 is a common marker for hematopoietic immune cells and CD31 is the marker for endothelial cells. CD140b is used to label the PDGFRβ+ cells. Therefore, DPP4+ APCs were sorted from CD45-CD31-PDGFRRβ+ cells with additional maker DPP4+ and DPP4- APCs were defined as CD45-CD31-PDGFRRβ+DPP4-. Details for subpopulation gating are described in Figure 2B.

23. Collect 10^5 sorted cells in each collection tube containing 200 μL flow buffer and keep sorted cells on ice.
24. Centrifuge at 600 × *g* for 5 min at 4°C.
25. Wash by resuspending cell pellets in ice-cold DPBS and centrifuge again at 600 × *g* for 5 min at 4°C. Discard the supernatant and keep the pellets.
26. Repeat the wash 3 times. Keep the pelleted cells at −80°C until use.

Protein extraction and mass spectrometry sample preparation

Timing: 2 days

⚠ CRITICAL: Wear gloves, clean lab coat, face mask, and use clean plasticware and equipment to minimize any contamination from keratin.

Samples are prepared for mass spectrometry following the Phase Transfer Surfactant (PTS) method,[16,17] with modifications as follows:

27. Suspend cell pellets (10^5 cells) in 15 μL PTS protein extraction buffer and sonicate for on ice using a Bioruptor water bath sonicator on high power with five 1-min on/1-min off cycles. Afterwards, adjust the volumes to 20 μL with PTS protein extraction buffer.
28. Determine the protein concentration of each sample using a Pierce BCA Protein Assay Kit, with the following adjustments:
    a. Prepare the working reagent (WR) solution by mixing 1000 μL reagent A with 20 μL reagent B. A 50:1 ratio of reagent A to reagent B is as per the manufacturer's protocol, however, these volumes are smaller than the manufacturer's protocol, because a smaller volume of sample is used for quantification.
    b. Prepare a series of albumin standards by diluting the supplied albumin standard (2000 μg/mL) with water at the following concentrations (μg/mL): 0; 125; 250; 500; 750; 1000; 1500; and 2000.
    c. Aliquot 30 μL WR solution into 0.5 mL microcentrifuge tubes for the standards and samples.
    d. Add 2 μL PTS protein extraction buffer to the tubes with WR solution for the standard reactions.
    e. Add 2 μL water to the tubes with WR solution for the sample reactions.
    f. Add either 2 μL standard or 2 μL sample to the appropriate tubes and mix.
    g. Incubate at 37°C for 30 min.
    h. Measure the absorbance of 2 μL of each reaction at 562 nm using a NanoDrop for determination of the protein concentration.
29. Adjust the protein amounts and samples volumes in PTS protein extraction buffer, so that the volumes are 20 μL and the protein amounts are the same across samples with a protein amount up to 10 μg.
30. Add 2.5 μL 100 mM DL-dithiothreitol (DTT) to a final concentration of 11 mM DTT with incubation at 50°C for 30 min with shaking at 600 rpm in a ThermoMixer C to reduce cysteine-cysteine disulfide bonds.
31. Add 2.5 μL 400 mM iodoacetamide (IAA) to a final concentration of 40 mM IAA, followed with incubation in the dark for 30 min at room temperature to alkylate free thiol groups.
32. Add 4.0 μL 400 mM cysteine to a final concentration of 55 mM cysteine followed with incubation at RT for 10 min to quench the alkylation reactions.
33. Dilute the samples with 80.0 μL of 50 mM ammonium bicarbonate, i.e., a five-fold dilution of PTS buffer.
34. Add 200 ng lysyl endopeptidase (Lys-C), so the amount is at least 1:50 (w:w) enzyme:sample and mix.
35. Add 200 ng trypsin, so the amount is at least 1:50 (w:w) enzyme:sample, and mix.
36. Digest the samples for 14 h overnight at 37°C with shaking at 600 rpm.

37. After overnight digestion add 200 μL ethyl acetate. Mix the samples and spin them down.
38. Add 16.5 μL 10% (v/v) trifluoroacetic acid (TFA) to a final concentration of 0.5% (v/v) TFA to acidify the samples. Mix the samples vigorously for 5-min at RT.

Note: The pH of the samples can be confirmed to be between 2 to 3 with 1–2 μL sample using pH indicator paper.

39. Centrifuge the samples at 12,000 × *g* for 5 min at RT to separate the samples into two phases; an upper organic phase and lower aqueous phase.
40. Discard the upper phase containing detergent.
41. Dry the lower aqueous phase containing digested tryptic peptides using a centrifugal vacuum concentrator (miVac DUO Concentrator, -OH setting, without heating for about 1.5 h).
42. Desalt the samples with ZipTip C18, 0.6 μL Pipette Tips:

Note: According to manufacturer's instructions, the binding capacity of ZipTip C18, 0.6 μL pipette tips is typically ≤ 5.0 μg peptides.

a. Dissolve each tryptic peptide sample in 10 μL 0.1% (v/v) TFA, 3% (v/v) acetonitrile, 97% (v/v) water.
b. Condition the ZipTip C18 with 3 washes of 10 μL 100% acetonitrile
c. Equilibrate the ZipTip C18 with 3 washes of 10 μL 0.1% TFA.
d. Load the sample by slowly pipetting the sample up and down 10 times.
e. Desalt the sample with 5 washes of 10 μL 0.1% (v/v) TFA, 3% (v/v) acetonitrile, 97% (v/v) water.
f. Elute the samples into clean tubes twice with 10 μL 0.1% (v/v) TFA, 50% (v/v) acetonitrile, 50% (v/v) water.
g. Vacuum dry the desalted peptides (miVac DUO Concentrator, -OH setting, without heating for about 30 min).

Note: Other solid-phase extraction methods may be used in place of ZipTips, such as MonoSpin columns (GL Sciences, Japan), Pierce Peptide Desalting Spin Columns (Cat. No. 89851, Thermo Fisher), or StageTips,[18] however, these should be tested beforehand since different types of membrane can affect peptide recovery.

43. To prepare samples for generation of a spectral library, a Pierce High pH Reversed-Phase (HPRP) Fractionation Kit can be used:
    a. Take an aliquot from each of the tryptic peptide samples and combine them so that the peptide amount is at least 10 μg. (We usually do this after the individual samples have been measured, as described below Vacuum dry the combined sample to evaporate organic solvent.
    b. Dissolve the combined peptide sample with 300 μL 0.1% (v/v) TFA.
    c. Use the HPRP Fractionation Kit according to manufacturer's instructions: Collect eight fractions in solution with increasing acetonitrile concentration from 5% (v/v) to 50% (v/v) mixed with 0.1% (v/v) triethylamine in water.
    d. Afterwards, dry all fractions with vacuum centrifugation (miVac DUO Concentrator, -OH setting, without heating for about 2.5 h).

Mass spectrometry measurement

Timing: 3 h per sample

LC-MS/MS measurements can be made using a Q Exactive Plus Orbitrap Mass Spectrometry System or other suitable equipment. At least three biological replicates for each sample type should be

prepared and measured. The following description applies to a Q Exactive Plus Orbitrap Mass Spectrometer with a Nanospray Flex ion source and an EASY-nLC 1200 liquid chromatography system.

44. Conditions common to measurements with data-independent acquisition (DIA) and data-dependent acquisition (DDA):
    a. An Acclaim PepMap 100 trap column with 3-μm C18 particles, inner diameter of 75-μm, and a filling length of 2-cm to concentrate and further desalt the tryptic peptides. Usually, we measure about 600 ng tryptic peptides per sample, although 400–1000 ng should generate data of sufficient quality and quantity.
    b. An analytical column with 3-μm C18 particles, 75-μm inner diameter and 12-cm filling length (Nikkyo Technos, Co., Ltd., Japan).
    c. LC/MS grade solvents: LC solvent A consists of 0.1% (v/v) formic acid in water and LC solvent B consists of 0.1% (v/v) formic acid, 80% (v/v) acetonitrile in water.
    d. The mass spectrometer ion transfer tube temperature is 250°C with 2.0 kV spray voltage.
    e. A flow rate of 300 nL/min is used with a 2-h gradient: 2% solvent B to 34% solvent B in 108 min, 34% solvent B to 95% solvent B in 2 min, with a final wash at 95% solvent B for 10 min, i.e., Gradient Information:

Time (mm:ss)	Duration (mm:ss)	Flow (nL/min)	Mixture, %B (0.1% formic acid, 80% acetonitrile, 20% water)
00:00	00:00	300	2.00
108:00	108:00	300	34.00
110:00	02:00	300	95.00
120:00	10:00	300	95.00

Note: the length of gradient can be adjusted according to the sample, e.g., a shorter gradient can be used for less complex samples.

45. For DIA measurements:
    a. If the tryptic peptides are prepared from $\leq 10^5$ cells, then dissolve in about 10 μL of 0.1% (v/v) formic acid, 3% (v/v) acetonitrile, 97% (v/v) water with sonication for 1 min. The peptide concentrations can be quantified here using a Pierce Quantitative Colorimetric Peptide Assay Kit. The following is according to the manufacturer's User Guide, but with the volumes scaled down to save precious samples:
        i. Mix 250 μL Colorimetric Peptide Assay Reagent A, 240 μL Colorimetric Peptide Assay Reagent B, and 10 μL Colorimetric Peptide Assay Reagent C to make the working reagent (WR).
        ii. Pipette 18 μL WR into 0.5 mL microcentrifuge tubes for the standards and samples.
        iii. Prepare a series of digest standards by diluting the supplied digest standard (1000 μg/mL) with water at the following concentrations (μg/mL): 0; 15.6; 31.3; 62.5; 125; 250; 500; and 1000.
        iv. Add 2 μL sample, 2 μL standard, or 2 μL water for the blank.
        v. Incubate at 37°C for 15 min.
        vi. Measure the absorbance of 2 μL of each reaction at 480 nm using a NanoDrop for determination of the peptide concentration.
        vii. The sample concentrations should be adjusted to a similar concentration. We usually inject 3 μL with 0.2 μg/μL sample for 600 ng peptide.

Note: We have found that 400–1000 ng is a suitable range of peptide amount for DIA analysis using this protocol. The chromatograms should always be examined to make sure that the MS1 and MS2 TIC signal intensities are similar across samples, and the signal profile is similar. Also, software (e.g., Spectronaut) used to analyze the DIA data can apply

normalization, and the graphs displaying the normalization should be checked. Also, since the sample volumes put in the MS vials are only around 8 μL, the volume should be checked to make sure that evaporation is not significant and additional solvent should be added as required.

b. The following MS parameters are applied:
   i. Data are acquired with 1 full MS and 32 overlapping isolation windows constructed covering the precursor mass range of 400–1200 m/z.
   ii. For full MS, resolution is set to 70,000, AGC target is set to $5e^6$, and the maximum injection time (IT) is set to 120 ms.
   iii. DIA segments are acquired at a resolution of 35,000 with an AGC target of $3e^6$ and an automatic maximum IT. The first mass is fixed at 150 m/z. HCD fragmentation is set to normalized collision energy of 27%.

46. For DDA measurements to generate a spectral library:
   a. If about 10 μg tryptic peptides are fractionated with HPRP fractionation, dissolve each fraction in 7.0 μL of 0.1% (v/v) formic acid, 3% (v/v) acetonitrile, 97% (v/v) water and inject 5.0 μL for measurement. Alternatively, determine the peptide concentration with a Pierce Quantitative Colorimetric Peptide Assay Kit, and inject 600 ng peptides from each fraction for measurement.
   b. The following MS parameters are applied:
      i. Full MS spectra are acquired from 380 to 1500 m/z at a resolution of 70,000, the AGC target of $3e^6$ and a maximum IT of 100 ms.
      ii. MS2 scans are recorded for the top 20 precursors at a resolution of 17,500; an AGC target of $1e^5$; a maximum IT of 60 ms; normalized collision energy of 27%; intensity threshold is set to $1.3e^4$; charge states 2–5 are included; and the dynamic exclusion is set to 20 s.

Note: To minimize and compensate for batch effects, the samples should ideally be measured using the same analytical column, with calibration of the mass spectrometer prior to measurement of samples. Quality control samples (e.g., Pierce HeLa protein digest standard, Thermo Fisher Cat # 88328) should be measured at the beginning and end of the sequence. The sample order should be randomized before putting the MS vials into the liquid chromatography system. Also, we recommend that at least two standard samples should be measured at the beginning and end of the loading sequence for every batch of samples measured, which can be a combined sample or technical replicates of designated samples, to assess for batch effects.

EXPECTED OUTCOMES

The methods to isolate adipocyte progenitor populations were reported in previous studies.[1,5,13,19] The quantities of cells collected via FACS sorting are dependent on the age, sex and adiposity of mice. For instance, about 60,000 FIPs, 25,000 APCs from gonadal WAT and 40,000 DPP4+ APCs and 30, 000 DPP4- APCs from inguinal WAT could be sorted out from two WAT depots of an 8-weeks-old lean male mouse (body weight between 20−25 *g*), respectively; for an 8-week-old lean female mouse (body weight between 20−25 *g*), about 40,000 FIPs and 15,000 APCs from gonadal WAT, 30,000 DPP4+ APCs and 20, 000 DPP4- APCs from inguinal WAT could be sorted out from two WAT depots, respectively. For mice after 16 HFD feeding (body weight > 40 *g*), over 120,000 FIPs and less than 6,000 APCs from gonadal WAT, about 75,000 DPP4+ APCs and 45, 000 DPP4- APCs from inguinal WAT could be harvested from 2 depots of an individual mouse. Ideally, >90% of sorted cells are viable after FACS sorting and are able to adhere and grow after seeding (Figure 3). For adipocyte progenitor cells that we used in our study, we recommend a total amount of > 20,000 cells to be collected for each sample. For cell types that have more protein content per cell, less numbers of cells may be needed. A few thousand unique proteins are expected to be quantified. For instance, we were able to quantitatively determine the relative abundances of

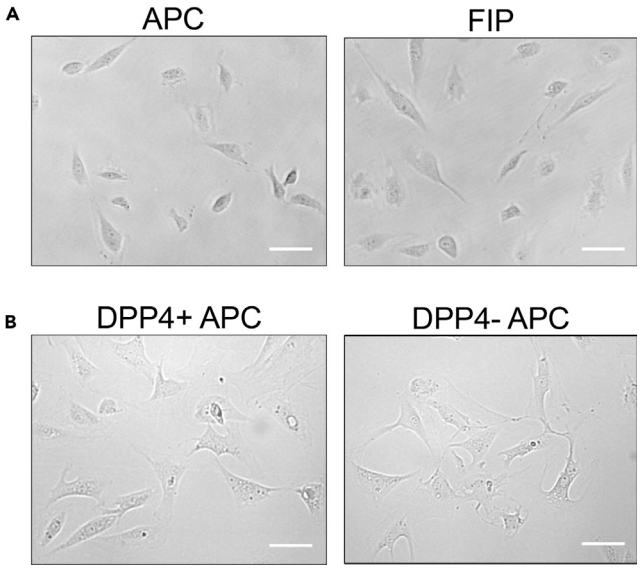

Figure 3. Bright field images of isolated adipose progenitor populations in culture

(A) Bright field images of cultured FIPs and APCs isolated from mouse gonadal WAT. Scale bar, 50 μM.

(B) Bright field images of cultured DPP4+ APCs and DPP4- APCs isolated from mouse inguinal WAT. Scale bar, 50 μM.

5021 proteins across 12 individual samples with high reproducibility among the replicates within each adipose tissue progenitor subpopulation groups (average coefficient of variation = 15.5%) (Figure 4A). Principal component analysis (PCA) of quantitative proteomics data revealed that the 4 adipose tissue progenitor subpopulations are evidently separated from one another at the protein level (Figure 4B). Unsupervised clustering of the quantified results highlighted anatomical origin and distinct functionality of the indicated adipose tissue progenitor subpopulations at the protein level (Figure 4C). Comparison between proteins quantified in replicates of male APCs and FIPs identified significantly regulated genes (674 up-regulated genes in APCs and 492 down-regulated genes in APCs) at the protein level, using cutoff values of fold change > 1.5, Q value < 0.05 (Figure 4D).

QUANTIFICATION AND STATISTICAL ANALYSIS

The 8 raw data files obtained from DDA of the HPRP-fractionated tryptic peptide fractions are analyzed together using Proteome Discoverer version 2.4 (Thermo Fisher Scientific, USA) with the UniProt reviewed *Mus musculus* (taxon 10090) database to generate a single results file. The following settings are applied: digestion enzyme specificity set to Trypsin (Full), precursor and fragment mass tolerances set to 10 ppm and 0.02 Da, respectively. Carbamidomethylation of cysteine is set as a static modification, oxidation of methionine, protein N-terminal acetylation, methionine loss, and acetylation and methionine loss are set as dynamic modifications. Up to 2 missed cleavages are allowed. The minimum peptide length is set to 6 and a minimum number of peptide sequences is set to 1. A concatenated decoy database is employed to calculate the false discovery rate (FDR). Search results are filtered with FDR 0.01 at both peptide and protein levels.

The filtered output from Proteome Discoverer is used to generate a sample-specific spectral library using Spectronaut software (Biognosys, Switzerland). Raw data files from DIA measurement are used for extraction of protein quantities with the generated spectral library. FDR is estimated with the mProphet approach, and a cutoff is set to 0.01 at both peptide precursor level and protein level.[20,21] Data filtering parameters for quantification can be set to Q-value percentile fraction 0.2 with global imputing, and cross run normalization with global normalization on the median. For differential abundance testing, an unpaired two-sample Student's t-test is performed. A multiple testing is performed to calculate the Q value (corrected p value). The table of significantly changed proteins is generated with a cutoff of Q value 0.05 and absolute log2ratio 0.58.

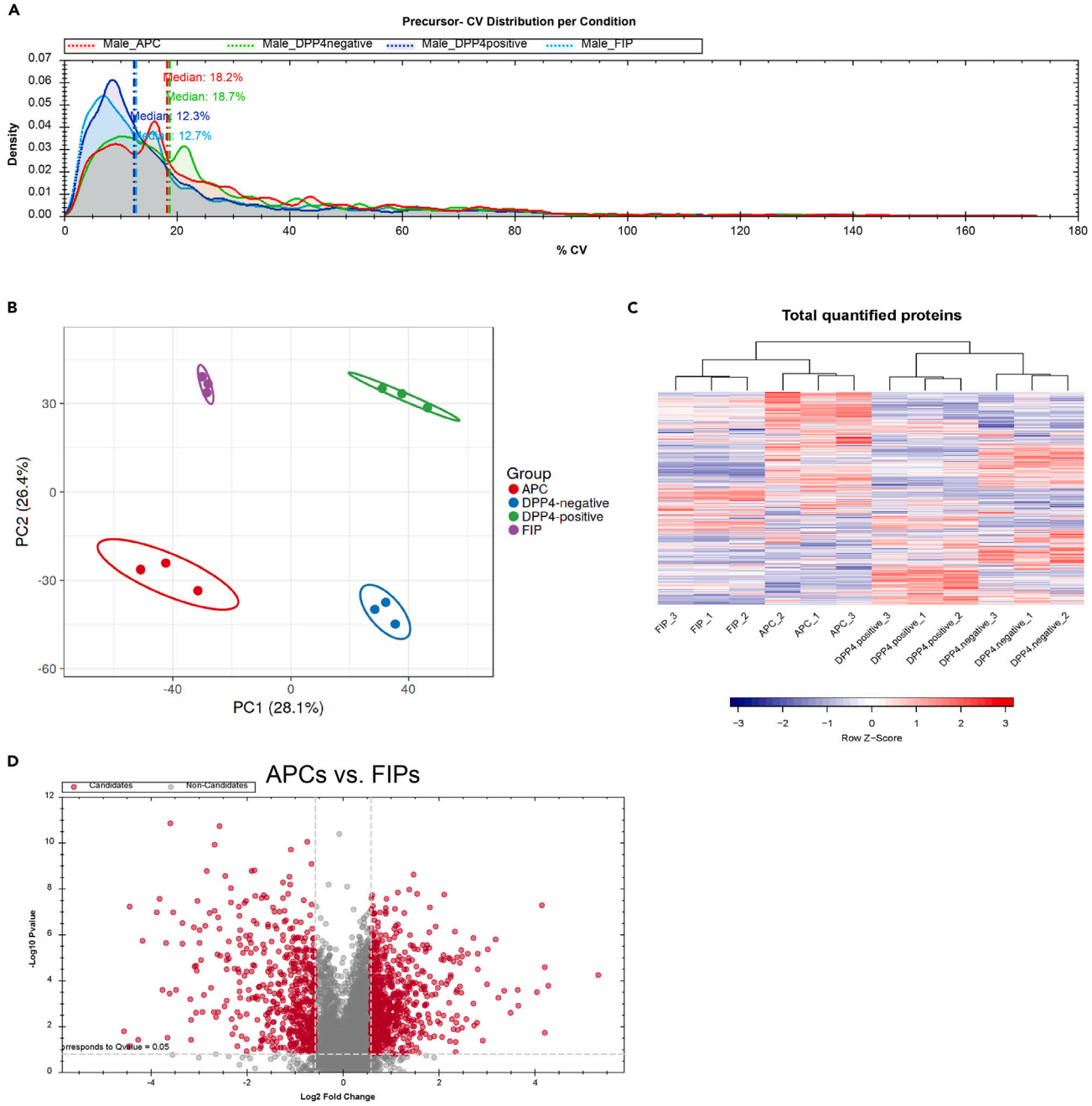

Figure 4. Quantitative proteomics data revealed distinct protein signatures of adipose tissue progenitor subpopulations

(A) Coefficient of variation (CV) of protein quantification across replicates of the 4 progenitor subpopulations.

(B) Principal component analysis based on quantitative proteomics data of the 4 progenitor subpopulations.

(C) Heatmap depicting protein levels across 12 male samples. Data were normalized to total abundance in each sample and z-score transformed. Red represents a z-score larger than 0 and blue represents a z-score smaller than 0.

(D) Volcano plot showing differences between male APCs and FIPs at the protein level. Proteins with significantly different abundances (corresponding to fold change > 1.5, Q value < 0.05) are shown in red color.

LIMITATIONS

This protocol is based on isolated adipocyte progenitor cells from depots in male mice, applications can be adapted to other adipose depots (such as brown adipose tissue) or adipose tissue from

female animals with minor but necessary modifications for optimal viability and quantity. Besides, the extensive tissue processing including collagenase digestion is a noteworthy caveat which may impact the proteomic profiles of the cells examined. Nevertheless, as described and discussed in the prior study,[1] overall expression profiles are retained to distinguish the heterogeneous nature of distinct adipose progenitor populations following this isolation procedure.

TROUBLESHOOTING

Problem 1

Cells have low sorting yields.

Potential solution

Flat-bottomed microcentrifuge tubes can increase the quantity of cells recovered with centrifugation. Also, ensure that the cells are washed with DPBS, otherwise contaminating protein from flow buffer may affect the final protein amount, especially when cell numbers are low.

Problem 2

Sorted cells have low viability.

Potential solution

Keeping the digested and filtered samples on ice during the entire procedure can increase cell viability. Also, resuspending cells from pellets with gentle pipetting is critical to increase cell viability.

Problem 3

Insufficient amounts of peptides for HPRP fractionation, e.g., when the project consists of samples of small protein/peptide amount.

Potential solution

If there is an insufficient peptide amount for HPRP fractionation, DIA data can be interpreted without a spectral library using different approaches, including DIA-Umpire, Group-DIA and directDIA, etc.[22]

Problem 4

Low numbers of proteins quantified with DIA analyses.

Potential solution

- Measure a QC sample, such as a Thermo Fisher HeLa digest (Cat. No. 88328). If the QC fails, then maintenance of the mass spectrometer is required.
- If the mass spectrometry condition is good, then the peptide amount should be quantified before loading, for example, using a Pierce Quantitative Colorimetric Assay Kit (Cat. No. 23275). Usually, 400–1000 ng peptides will give a good dynamic range for protein quantification with DIA using a Q Exactive Plus mass spectrometer. If the peptide amount for injection is significantly below 400 ng, the sample preparation procedure should be done with a control sample to find out the source of the sample loss.
- If an adequate amount of peptide is injected, yet the number of proteins identified is still low, then there may be contamination in the samples. Alongside the species-specific proteome database FASTA file, a database for contaminants should be included in the data analysis. For example, databases of common contaminants are maintained by the Max Planck Institute of Biochemistry, Martinsried (http://www.coxdocs.org/doku.php?id=maxquant:start_downloads.htm) or The Global Proteome Machine Organization common Repository of Adventitious Proteins (https://www.thegpm.org/crap/). Depending upon the level of contamination, the washing of the samples during preparation may need to be optimized.

- Also, the peptide missed cleavage level determined with Spectronaut software should typically be about 20% with this sample preparation protocol. If the peptide missed cleavage level is significantly higher, it suggests an issue with the protein digestion, and the enzyme and reagent quality should be checked.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Yibo Wu (wulabproteomics@gmail.com).

Materials availability

New materials and reagents generated in this study are available upon request to the lead contact with a complete Material Transfer Agreement.

Data and code availability

This study did not generate new code. The mass spectrometry datasets (available at ProteomeXchange under the accession number PXD023829) were previously published[1] and re-analyzed with the permission from Cell Metabolism.

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AUTHOR CONTRIBUTIONS

B.S., M.S., and Y.W. conceptualized the study; B.S., C.S.B., H.T., X.Z., and Y.P. performed the experiments; and B.S., C.S.B., M.S., and Y.W. analyzed the data, prepared the figures, and wrote the original draft. R.K.G. and Y.W. revised the manuscript. All authors discussed and approved the final manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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