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# Isolation of Adipogenic and Fibro-inflammatory Stromal Cell Subpopulations from Murine Intra-abdominal Adipose Depots

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Cover letter

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Department of Internal Medicine
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July 10th, 2020

Vineeta Bajaj, Ph.D.
Review Editor
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Dear Dr. Bajaj,

Enclosed, please find our **revised manuscript** entitled, ***"Isolation of Adipogenic and Fibro-inflammatory Stromal Cell Subpopulations from Murine Intra-abdominal Adipose Depots,***" which Dr. Myers kindly invited to submit as part of the special series ***"State-of-the-art methods to study adipocytes and adipose tissue."***

Here, we present a protocol that describes the technical approach to isolate adipogenic and fibro-inflammatory stromal cell subpopulations from murine intra-abdominal WAT depots by fluorescence-activated cell sorting or immunomagnetic bead separation.

We have attempted to address the major concerns of Reviewers 2 and 3 through additions to the protocol, an addition of a new table, and revision of the Discussion section.

We greatly appreciate the opportunity to contribute to this special series. We look forward to hearing your feedback on the paper.

Sincerely,

Rana K. Gupta
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Touchstone Diabetes Center

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**TITLE:**

# Isolation of Adipogenic and Fibro-Inflammatory Stromal Cell Subpopulations from Murine Intra-Abdominal Adipose Depots

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**KEYWORDS:**

white adipose tissue, preadipocyte, adipocyte precursor cells, adipogenesis, stromal vascular fraction, cell isolation, magnetic bead separation, visceral adipose tissue

**SUMMARY:**

This protocol describes the technical approach to isolate adipogenic and fibro-inflammatory stromal cell subpopulations from murine intra-abdominal white adipose tissue (WAT) depots by fluorescence-activated cell sorting or immunomagnetic bead separation.

**ABSTRACT:**

The stromal-vascular fraction (SVF) of white adipose tissue (WAT) is remarkably heterogeneous and consists of numerous cell types that contribute functionally to the expansion and remodeling of WAT in adulthood. A tremendous barrier to studying the implications of this cellular heterogeneity is the inability to readily isolate functionally distinct cell subpopulations from WAT SVF for in vitro and in vivo analyses. Single-cell sequencing technology has recently identified functionally distinct fibro-inflammatory and adipogenic PDGFRβ+ perivascular cell subpopulations in intra-abdominal WAT depots of adult mice. Fibro-inflammatory progenitors (termed, “FIPs”) are non-adipogenic collagen producing cells that can exert a pro-inflammatory phenotype. PDGFRβ+ adipocyte precursor cells (APCs) are highly adipogenic both in vitro and in

vivo upon cell transplantation. Here, we describe multiple methods for the isolation of these stromal cell subpopulations from murine intra-abdominal WAT depots. FIPs and APCs can be isolated by fluorescence-activated cell sorting (FACS) or by taking advantage of biotinylated antibody-based immunomagnetic bead technology. Isolated cells can be used for molecular and functional analysis. Studying the functional properties of stromal cell subpopulation in isolation will expand our current knowledge of adipose tissue remodeling under physiological or pathological conditions on the cellular level.

**INTRODUCTION:**

White adipose tissue (WAT) represents the principal site for energy storage in mammals. Within this tissue, adipocytes, or "fat cells," store excess calories in the form of triglyceride, packaged into large unilocular lipid droplets. Moreover, adipocytes secrete a multitude of factors that regulate various aspects of energy homeostasis[1-3]. Adipocytes constitute the bulk of WAT volume; however, adipocytes only represent less than 50% of total cells found in WAT[4,5]. The non-adipocyte compartment of WAT, or stromal-vascular fraction (SVF), is quite heterogeneous and contains vascular endothelial cells, tissue-resident immune cells, fibroblasts, and adipocyte precursor cell (APC) populations.

WAT is exceptional in its remarkable capacity to expand in size as the demand for energy storage increases. Maintaining this tissue plasticity is essential as adequate storage of lipids in WAT protects against deleterious ectopic lipid deposition into non-adipose tissues[6]. The manner by which individual WAT depots undergo this expansion in response to caloric excess is a critical determinant of insulin sensitivity in the setting of obesity[7]. Pathologic WAT expansion, observed in obese individuals with metabolic syndrome, is characterized by preferential expansion of visceral WAT depots at the expense of metabolically favorable subcutaneous fat tissue. Moreover, insulin resistance in obesity is associated with pathologic remodeling of WAT. This is characterized by hypertrophic growth of existing adipocytes (increase in size), inadequate angiogenesis, chronic metabolic-inflammation, accumulation of extracellular matrix components (fibrosis), and tissue hypoxia[8,9]. These WAT phenotypes of obesity are associated with hepatic steatosis and insulin resistance, similar to what is observed in the condition of lipodystrophy (absence of functional WAT). In contrast, healthy WAT expansion is observed in the metabolically healthy obese population and is characterized by preferential expansion of protective subcutaneous WAT and depot expansion through adipocyte hyperplasia[10]. The recruitment of new adipocytes is mediated by de novo adipocyte differentiation from adipocyte precursor cells (APCs) (termed, "adipogenesis"). Adipocyte hyperplasia coincides with relatively lower degrees of WAT fibrosis and metabolic inflammation[6,11]. A multitude of cell types within the WAT microenvironment directly influence the health and expandability of WAT in obesity[12]. As such, defining the function of the various cell types present in WAT remains a high priority for the field.

Over the past decade, several strategies have been employed to define and isolate native APCs from human and mouse WAT SVF[13]. Such strategies isolate APCs based on the cell surface expression of common mesenchymal stem/progenitor cell markers using antibody-based cell separation techniques. These approaches include fluorescence-activated cell sorting (FACS), using fluorophore-labelled antibodies, or immunomagnetic bead separation (i.e., chemically

modified antibodies). Cell surface proteins targeted for the isolation of APCs include PDGFRα, PDGFRβ, CD34, and SCA-1. These approaches have helped enrich for APCs; however, cell populations isolated based on these markers are quite heterogeneous. Very recent single-cell RNA-sequencing (scRNA-seq) studies have highlighted the molecular and functional heterogeneity of stromal cells within the isolated stromal-vascular fraction (SVF) of murine WAT[14-17]. From our own scRNA-seq and functional analyses, we have identified and characterized functionally distinct immune-modulating and adipogenic PDGFRβ+ perivascular cell subpopulations in the stromal compartment of intra-abdominal WAT in adult mice[15]. Fibro-inflammatory precursors, or FIPs, represent a prominent subpopulation of PDGFRβ+ cells and can be isolated based on LY6C expression (LY6C+ PDGFRβ+ cells)[15]. FIPs lack adipogenic capacity, exert a strong pro-inflammatory response to various stimuli, produce collagen, and secrete anti-adipogenic factors[15]. The pro-inflammatory and fibrogenic activity of these cells increases in association with obesity in mice, implicating these cells as regulators of WAT remodeling. The LY6C- CD9- PDGFRβ+ subpopulation represents adipocyte precursor cells (APCs). These APCs are enriched in the expression of *Pparg* and other pro-adipogenic genes, and readily differentiate into mature adipocytes in vitro and in vivo[15]. Here, we provide a detailed protocol for the isolation of these distinct cell populations from intra-abdominal WAT depots of adult mice using FACS, and immunomagnetic bead separation with biotinylated antibodies. This protocol can be used to isolate functionally distinct adipose progenitor subpopulations from multiple intra-abdominal WAT depots of adult male and female mice[15]. Studying these functionally distinct cell populations in isolation may contribute greatly to our current understanding of the molecular mechanisms that regulate adipogenesis and intra-abdominal adipose tissue remodeling in health and disease.

The protocol below details the isolation of adipose progenitors from murine epididymal WAT; however, the same procedure can be used to isolate corresponding cells from the mesenteric and retroperitoneal WAT depots of both male and female mice[15]. A detailed protocol on how to identify and isolate these depots in mice can be found in Bagchi et al.[18]. This protocol has been optimized for the use of mice 6-8 weeks of age. The frequency and differentiation capacity of APCs may decline in association with ageing.

**PROTOCOL:**

All animal protocols and procedures have been approved by the University of Texas Southwestern Medical Center Institutional Animal Use and Care Committee.

## 1. Isolation of stromal vascular fraction (SVF) from gonadal white adipose tissue

1.1. Dissect the gonadal white adipose tissue from 6-8-week-old mice and place fat pads in 1x PBS solution.

1.2. Combine up to 4 fat depots (2-4 depots from 1-2 mice recommended) and mince the tissue in a 10 mL beaker containing 200 µL of digestion buffer (1x HBSS, 1.5% BSA and 1 mg/mL collagenase D).

1.3. Transfer the minced tissue to a 50 mL centrifuge tube containing 10 mL digestion buffer.

1.4. Incubate mixture at 37 °C in a water bath for 1 h while shaking.

1.5. Filter the digested tissue through a 100 µm cell strainer to remove undigested tissue.

1.6. Dilute filtered sample to 30 mL with PBS containing 2% FBS, and centrifuge at 600 *x g* for 5 min at 4 °C. Aspirate the supernatant, which contains mature adipocytes.

1.7. Proceed immediately to preferred cell isolation method.

**2. Isolation of APCs and FIPs using FACS**

2.1. Dissolve the pellet in 1 mL of 1x RBC lysis buffer by shaking the tube gently.

2.2. Incubate at RT for 1-2 min.

2.3. Add 10 mL of 2% FBS/PBS and pass through a 40 µm cell strainer into a clean 50 mL centrifuge tube.

2.4. Centrifuge at 600 *x g* for 5 min at 4 °C.

2.5. Aspirate media and resuspend the pellet in 400-800 µL of 2% FBS/PBS containing Fc block (1:200).

2.6. Incubate at 4 °C for 10 min.

2.7. Transfer 400 µL-800 µL of cell suspensions containing ≤$10^6$ cells per mL to 1.5 mL tubes and add antibodies. Antibody concentrations are listed in the **Table of Materials** section.

2.7.1. Prepare separate control tubes for 1) unstained cells, 2) single color controls, and 3) FMO (fluorescence minus one) controls.

2.7.2. Stain the samples with PDGFRβ, CD45, CD31, CD9, LY6C antibodies.

2.8. Incubate at 4 °C for 15 min protected from light.

2.9. Centrifuge at 600 *x g* for 5 min at 4 °C.

2.10. Aspirate media and resuspend cells in 400 µL of 2% FBS/PBS.

2.11. Centrifuge at 600 *x g* for 5 min at 4 °C.

2.12. Aspirate media and resuspend cells in 400-800 µL of 2% FBS/PBS. Then pass through 40 µm filter caps into 5 mL polystyrene round-bottom tubes for FACS.

2.13. Follow the following steps for gating.

2.13.1. Use unstained and single-color controls for compensation.

2.13.2. Use FMO controls to set experimental gates.

2.13.3. Use the gating strategy shown in **Figure 1** to obtain APCs and FIPs. Gate APCs as $CD45^-$ $CD31^-$ $PDGFR\beta^+$ $LY6C^-$ $CD9^-$ and FIPs as $CD45^-$ $CD31^-$ $PDGFR\beta^+$ $LY6C^+$ cells.

2.14. Collect sorted cells in tubes containing 500 µL of 100% serum and maintain the cells on ice.

2.15. Centrifuge cells at 600 *x g* for 5 min at 4 °C. Add 2% FBS/PBS to wash the cells by centrifuging again at 600 *x g* for 5 min at 4 °C. Discard the supernatant use the pelleted cells.

2.16. Resuspend the pelleted cells in the appropriate volume of gonadal APC culture media (for cell culture) or appropriate buffer for subsequent analysis.

## 3. Immunomagnetic separation of adipogenic and non-adipogenic fractions

3.1. Isolate SVF from gonadal white adipose tissue following steps 1.1-1.7.

3.2. Aspirate the supernatant and resuspend the SVF pellet in 10 mL of 1x commercially available MS Buffer.

3.3. Filter the cell suspension through a 40 µm cell strainer.

3.4. Centrifuge at 600 *x g* for 5 min at 4 °C.

3.5. Aspirate the supernatant and resuspend pelleted cells in 100 µL of 1x MS Buffer.

3.6. Perform $CD31^+$ and $CD45^+$ cell depletion as discussed below (**Figure 2A, Step 1**). This is done to remove endothelial and hematopoietic lineage cells.

3.6.1. Count cells using a cell counter and adjust the concentration to ≤ 1 x $10^8$ cells/mL.

3.6.2. Add the biotin conjugated CD31 and CD45 antibodies to the cell suspension, each at a concentration of ≤ 0.25 µg per $10^6$ cells and incubate on ice for 15 min.

3.6.3. Wash cells by adding 4 mL of 1x MS Buffer.

3.6.4. Centrifuge at 600 *x g* for 5 min at 4 °C.

3.6.5. Aspirate the supernatant and resuspend the pellet with 100 µL of 1x MS buffer.

3.6.6. Add 10 µL of streptavidin nanobeads and incubate cells on ice for 15 min.

3.6.7. Wash cells by adding 4 mL of 1x MS buffer.

3.6.8. Centrifuge at 600 *x g* for 5 min at 4 °C, then aspirate the supernatant.

3.6.9. Resuspend cells in 2.5 mL of 1x MS buffer and transfer to a 5 mL polypropylene tube.

3.6.10. Place the tube in the magnet rack for 5 min.

3.6.11. Collect unlabeled cells by pouring the cell suspension into a clean 15 mL centrifuge tube.

NOTE: Avoid shaking or blotting off hanging droplets as this leads to contamination from unwanted cell fraction.

3.6.12. Remove the tube from magnet and repeat steps 3.6.9. to 3.6.11. two more times for a total of 3 washes.

3.7. Separation of adipogenic and non-adipogenic fractions (**Figure 2A, Step 2**)

3.7.1. Continue working with unlabeled fraction (i.e., $CD45^-$ $CD31^-$ fraction).

3.7.2. Centrifuge the 15 mL tube containing unlabeled cells at 600 *x g* for 5 min at 4 °C.

3.7.3. Aspirate the supernatant and resuspend the pellet in 100 µL of 1x MS buffer.

3.7.4. Add biotin conjugated LY6C and CD9 antibodies, respectively, at a concentration of ≤ 0.25 µg per $10^6$ cells and incubate on ice for 15 min.

3.7.5. Follow steps 3.6.3. to 3.6.12. to complete the second separation.

3.7.6. Collect unbound fractions. Unlabeled cells ($LY6C^-$ $CD9^-$) represents adipogenic fraction containing APCs (**Figure 2A, Step 3**).

3.7.7. Remove tube from the magnet, resuspend and elute bound cells in 1x MS buffer. Eluates or labeled fraction ($LY6C^+$ $CD9^+$), represents non-adipogenic fraction containing FIPs (**Figure 2A, Step 3**).

3.7.8. Centrifuge labeled and unlabeled fractions at 600 *x g* for 5 min to pellet cells at 4 °C.

3.8. Proceed immediately to cell culture (step 6) or use undifferentiated precursors for preferred analyses (step 4 and/or step 5).

**4. Assess purity of adipogenic and non-adipogenic fractions by flow cytometry**

4.1. Resuspend bead-isolated cell fractions in 200-400 µL of 2% FBS/PBS containing Fc block (1:200).

4.2. Incubate at 4 °C for 10 min.

4.3. Transfer the cell suspension to 1.5 mL tubes and add antibodies. Antibody concentrations are listed in the **Table of Materials**.

4.3.1. Prepare separate control tubes for 1) unstained cells, 2) single color controls, and 3) FMO controls.

4.3.2. To the samples, add CD45, CD31, CD9, and LY6C antibodies.

4.4. Incubate at 4 °C for 15 min protected from light.

4.5. Centrifuge at 600 *x g* for 5 minutes at 4 °C.

4.6. Aspirate supernatant and resuspend cells in 400 µL of 2% FBS/PBS.

4.7. Centrifuge the suspension at 600 *x g* for 5 min at 4 °C.

4.8. Aspirate the supernatant and resuspend cells in 400-800 µL of 2% FBS/PBS. Filter through 40 µm filter caps into 5 mL polystyrene round-bottom tubes for analysis by flow cytometry.

4.9. Flow cytometry analysis to assess purity

4.9.1. Use unstained and single-color controls for compensation.

4.9.2. Use FMO controls to set experimental gates.

4.9.3. Use the gating strategy for live and single cells as shown in **Figure 2B**.

4.9.4. Perform the gating as shown in **Figure 2B** for CD45, CD31, LY6C, and CD9.

4.9.5. Use flow cytometry software to evaluate the frequencies of $CD45^-$ $CD31^-$ $LY6C^-$ $CD9^-$ cells (APCs) and $CD45^-$ $CD31^-$ $LY6C^+$ cells (FIPs) within isolated fractions.

**5. Gene expression analysis using quantitative PCR to assess purity of FIPs and APCs**

5.1. Extract mRNA from magnetically or FACS isolated cells using commercially available extraction kit (see **Table of Materials**) following the manufacturer's instructions.

5.2. Use 1 µg of RNA to synthetize cDNA using a cDNA Reverse Transcriptase kit (see **Table of Materials**) by following the manufacturer's instructions.

5.3. Determine relative mRNA levels of APC and FIPs-selective genes (**Table 1**) by quantitative PCR using SYBR green PCR master mix.

5.3.1 Set up the sample reaction for PCR as follows: 5 µL of 2x SYBR green dye, 1 µL of cDNA (~50 ng/ µL), 0.5 µL of each forward and reverse primer (10 µM) and 3 µL of water.

5.3.2 Use the following standard PCR conditions for the quantitative PCR run: hold stage: 50 °C for 2 min, 95°C for 10 min; PCR stage (40 cycles): 95 °C for 15 s, 60 °C for 1 min.

5.4. Normalize values to house-keeping gene (*Rps18*) levels by calculating ΔΔ-Ct.

5.5. To evaluate statistical significance, perform unpaired Student's t-test.

## 6. Cell culture and differentiation

6.1. Centrifuge magnetically- or FACS- isolated cells at 600 *x g* for 5 min at 4 °C.

6.2. Resuspend the pellet in 500 µL of gonadal APC culture media and plate 40K cells/well from each fraction in a 48 well culture plate.

6.3. Replace media every 1-2 days. APCs will begin to undergo differentiation into adipocytes as they approach confluence. FIPs maintained in the same media are resistant to undergoing adipogenesis.

**REPRESENTATIVE RESULTS:**

This protocol describes two strategies that allow for the isolation of distinct stromal cell populations from intra-abdominal WAT depots of adult mice. APCs and FIPs can be isolated by FACS (**Figure 1**) or immunomagnetic bead separation with biotinylated antibodies (**Figure 2**). Both approaches utilize reagents and antibodies that are all commercially available. Immunomagnetic bead separation leads to the separation of adipogenic from non-adipogenic cells from the gWAT SVF. Flow cytometry analysis showed that 75% of cells within the adipogenic fraction represented LY6C$^-$ CD9$^-$ APCs. >75% of the non-adipogenic fraction represented FIPs (LY6C$^+$ cells).

[Place **Figure 1** and **Figure 2** here]

Light microscopy and gene expression analysis demonstrate that APCs isolated by FACs or through magnetic bead separation from gWAT of 6-8 week-old mice differentiated into lipid-

containing adipocytes to a high degree within 7-10 days following the initial plating of cells in Gonadal APC Culture media (**Figure 3**). In contrast, non-adipogenic precursors (such as fibro-inflammatory precursors, or FIPs) remained fibroblast-like and did not become adipocytes when maintained in the same culture media (Gonadal APC Culture Media) (**Figure 3**). It should be noted that few cells in the non-APCs cultures showed some lipid accumulation (**Figure 3D**). These likely arise from APC-contamination during cell isolation. Additional washes might improve the purity of this fraction.

[Place **Figure 3** here]

**FIGURE AND TABLE LEGENDS:**

**Table 1: qPCR primers sequences used to validate the isolation of FIPs and APCs**

**Figure 1: Isolation of PDGFRβ+ stromal cell subpopulations from gonadal WAT by FACS.** (**A**) Schematic overview of the procedure: The stromal vascular fraction (non-adipocyte cells) was separated from mature adipocytes by enzymatic tissue digestion and centrifugation. Fluorescence-activated cell sorting (FACS) was then used to remove endothelial ($CD31^+$) and hematopoietic ($CD45^+$) lineage cells and isolate $LY6C^+$ $PDGFR\beta^+$ cells (FIPs) and $LY6C^-$ $CD9^-$ $PDGFR\beta^+$ cells (APCs). (B) Representative FACS collection gates. Panel A is reproduced from ref.[11] with permission.

**Figure 2: Separation of adipogenic and non-adipogenic stromal cells by immunomagnetic bead separation.** (**A**) Schematic overview of the procedure: Step 1: $CD31^+$ & $CD45^+$ cells bind to the magnet. This removes both endothelial and hematopoietic lineage cells. The eluate containing $CD31^-$ & $CD45^-$ cells were collected and then incubated with antibodies recognizing LY6C and CD9, respectively. Step 2: $CD9^+$ and $LY6C^+$ cells binding to the magnet. Step 3: The supernatant (unbound fraction) containing CD9- & LY6C- cells was collected as this represented the adipogenic fraction (APCs). Non-adipogenic $CD9^+$ and $LY6C^+$ cells bound to nanospheres were eluted as the non-APC fraction containing FIPs. (**B**) Flow cytometry analysis to assess the frequency of APCs ($LY6C^-$ $CD9^-$) and FIPs ($LY6C^+$) within adipogenic and non-adipogenic fractions, respectively.

**Figure 3: In vitro differentiation of PDGFRβ+ stromal cell populations isolated from gWAT of adult mice.** (**A-D**) Representative bright-field images of differentiated stromal cell subpopulations isolated by immunomagnetic bead separation (**A-B**) or FACS (**C-D**) from 6-8-week-old mouse gWAT SVF. Images were taken seven days after plating cells in Gonadal APC Culture Media. Within 7-10 days of plating, APCs undergo spontaneous adipocyte differentiation. Magnification 10X. Scale = 250 μM. (**E-F**) mRNA levels of adipocyte-selective genes in differentiated cultures shown in A-D. Bar graphs represent mean + SEM.

**DISCUSSION:**

The C57BL/6 strain of mice is the most used mouse strain in studies of diet-induced obesity. C57BL/6 mice rapidly gain weight when placed on a high-fat diet (HFD) and develop some of the

prominent features of metabolic syndrome associated with obesity (e.g., insulin resistance and hyperlipidemia). Notably, WAT expansion occurring in association with high-fat diet (HFD) feeding occurs in a depot-specific manner[19-23]. Expansion of the subcutaneous inguinal WAT depot (iWAT) occurs almost exclusively through adipocyte hypertrophy, whereas the expansion of the gonadal WAT (gWAT) occurs through both adipocyte hypertrophy and hyperplasia. The gWAT depot of obese mice is also a prominent site of metabolic inflammation. Thus, the gWAT depot of diet-induced obese mice represents a model to study multiple features of adipose tissue remodeling linked to obesity.

Over the past several years, the most commonly used strategies to prospectively isolate APCs from adipose depots selects cells on the basis of CD29, SCA-1, and CD34 expression ($CD29^+$ $CD34^+$ $SCA\text{-}1^+$ $CD31^-$ $CD45^-$)[24,25]. This approach remains useful for the selection of APCs from iWAT and other WAT depots; however, in the gonadal WAT depot and other intra-abdominal depots, CD34 and SCA-1 expression are enriched in anti-adipogenic cells rather than APCs[15,26]. Buffolo et al. provide direct evidence of this, demonstrating that gWAT stromal cells selected on the basis of high expression of CD34 ($CD34^{high}$) are anti-adipogenic[26]. Therefore, the selection of cells based on the expression of CD34 and SCA-1 from this WAT depot yields a heterogeneous population that likely includes FIPs. The presence of such anti-adipogenic cells may explain the reported lack of adipogenic potential that isolated gonadal $CD34^+$ $SCA\text{-}1^+$ cells possess in vitro when compared to corresponding cells from the inguinal WAT depot[25]. The approach we describe here allows for the enrichment of intra-abdominal WAT APCs that are highly adipogenic in vitro. Moreover, APCs transplanted into lipodystrophic mice can form an ectopic fat pad[15]. Our strategy allows for the isolation of APCs from multiple intra-abdominal depots of both male and female mice, including gWAT, mesenteric WAT, and retroperitoneal WAT[15]. Multiple genetic lineage tracing studies demonstrate that adipocytes emerging in gWAT in association with HFD feeding originate from perivascular stromal cells expressing *Pdgfrb* (PDGFRβ protein)[21,23]. Importantly, the health of gWAT in obese mice is dependent on the adipogenic capacity of PDGFRβ+ cells[27]. These data support the notion that the gWAT APCs isolated by the approach described here are of physiological relevance.

Gonadal WAT APCs isolated by this approach differentiate rapidly into adipocytes upon reaching confluence in two-dimensional culture, or even prior to confluence. Unlike most established preadipocyte cell lines, these primary APCs do not require the addition of commonly used adipogenic factors (i.e., dexamethasone, IMBX, or PPARγ agonist). Readers should note that the commercially used ITS supplement used in this protocol does contain high levels of insulin. In addition, adipocyte differentiation can vary significantly with different sources/lots of FBS. Testing multiple lots of FBS is sometimes necessary to find serum that supports differentiation. Moreover, the varying levels of endotoxin in FBS may also influence the baseline pro-inflammatory phenotypes of APCs and FIPs.

FACS has been the commonly used technique for isolating APCs from the adipose SVF. This approach allows for the precise separation of cell populations and removal of debris and dead cells. Nevertheless, the duration and physical stress imposed on the cells may impact gene expression and/or cellular function. Moreover, multi-channel cell sorters may not be readily

available to all investigators. We provide details here on how APCs can be also isolated by magnetic bead separation. From our experience, the yield of both populations through magnetic bead separation is lower than observed when using FACS. Moreover, as shown in **Figure 2** above, this approach sacrifices some degree of purity. Nevertheless, magnetic bead separation yields cultures of APCs with high adipogenic potential. The purity of APC isolated through this approach can likely increase with repeated washing steps; however, this may compromise yield. A critical step in the protocol is the binding of biotinylated antibodies to target antigens on the surface of desired or undesired cells. If incubation time is insufficient or antibody concentrations are too low, target cells will remain unlabeled. Investigators may need to optimize antibody concentration for their isolations. The same principle applies to the incubation with streptavidin nanospheres. Failure to form streptavidin-biotin complexes will affect the efficiency of the isolation. Researchers should also pay additional attention to each washing step and resist the temptation to blot off any hanging droplets when collecting or discarding eluates from the magnet; blotting can result in cross-contamination from undesired cell populations. It should be of note that when performing a positive selection, the nanobeads are not removed from the cell fraction and may be detectable under a microscope. In our experience, the presence of these beads does not interfere with downstream functional assays. Investigators should note that under this magnetic-bead separation strategy, the non-APC fraction remains heterogeneous. This population contains mostly FIPs (>75% LY6C$^+$); however, other cell types are likely present (e.g., CD9$^+$ mesothelial cells). As such, the magnetic bead separation protocol presented here is perhaps most useful when the aim is to simply isolate APCs, and FACS machines are not readily available or cost prohibitive.

Investigators should also note important limitations to the overall sorting strategies described here. First and foremost, the protocols we describe here are currently only applicable to intra-abdominal WAT depots of mice. The subcutaneous iWAT depot in mice contains PDGFRβ+ adipose progenitors; however, LY6C expression does not readily discriminate between functionally distinct progenitor subpopulations within that depot[27]. Readers are referred to the work of Merrick et al. and Church et al. for protocols to isolate APCs from that depot[16,25]. We have not yet tested whether this sorting strategy can isolate functional APCs from rats. Moreover, it should be noted that there is no apparent ortholog for LY6C in humans[28]. As such, human APCs cannot be sorted based on these markers. Recent single-cell sequencing studies of human adipose stromal cells may lead to new strategies to isolate distinct progenitor populations from human tissues[29]. Second, there are limitations to the use of the cells in vitro. FIPs are highly proliferative and can be propagated for several passages and maintain their functional properties; however, APCs substantially lose their adipogenic potential with passage. This provides a technical challenge for those aiming to perform biochemical assays that require high cell numbers or manipulate gene expression (e.g., CRISPR/Cas9 or RNA interference). In our view, our approach is well-suited when the objective is to compare the frequency and properties of native APCs and/or FIPs derived directly from intra-abdominal WAT depots of animal models (e.g., APC frequency in control and experimental animals; comparison of male and female progenitors; effect of diet, age, etc., on progenitor frequency and properties). The ability to study these distinct cell populations in isolation should greatly aid in attempts to dissect molecular mechanisms that regulate adipogenesis and adipose tissue remodeling in mice.

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**DISCLOSURES:**

T.A.P. is an employee and shareholder in Novo Nordisk A/S

**REFERENCES:**

1 Ouchi, N., Parker, J. L., Lugus, J. J., Walsh, K. Adipokines in inflammation and metabolic disease. *Nature Reviews Immunology.* **11** (2), 85-97 (2011).

2 Rosen, E. D., Spiegelman, B. M. What we talk about when we talk about fat. *Cell.* **156** (1-2), 20-44 (2014).

3 Funcke, J. B., Scherer, P. E. Beyond adiponectin and leptin: adipose tissue-derived mediators of inter-organ communication. *Journal of Lipid Research.* **60** (10), 1648-1684 (2019).

4 Eto, H. et al. Characterization of structure and cellular components of aspirated and excised adipose tissue. *Plastic and Reconstructive Surgery.* **124** (4), 1087-1097 (2009).

5 Hirsch, J., Batchelor, B. Adipose tissue cellularity in human obesity. *Clinics in Endocrinology and Metabolism.* **5** (2), 299-311 (1976).

6 Ghaben, A. L., Scherer, P. E. Adipogenesis and metabolic health. *Nature Reviews Molecular Cell Biology.* **20** (4), 242-258 (2019).

7 Hepler, C., Gupta, R. K. The expanding problem of adipose depot remodeling and postnatal adipocyte progenitor recruitment. *Molecular Cell Endocrinology.* **445**, 95-108 (2017).

8 Jo, J. et al. Hypertrophy and/or Hyperplasia: Dynamics of Adipose Tissue Growth. *PLoS Computational Biology.* **5** (3), e1000324 (2009).

9 Sun, K., Kusminski, C. M., Scherer, P. E. Adipose tissue remodeling and obesity. *Journal of Clinical Investigation.* **121** (6), 2094-2101 (2011).

10 Kloting, N. et al. Insulin-sensitive obesity. *American Journal of Physiology-Endocrinology and Metabolism.* **299** (3), E506-515 (2010).

11 Vishvanath, L., Gupta, R. K. Contribution of adipogenesis to healthy adipose tissue expansion in obesity. *Journal of Clinical Investigation.* **129** (10), 4022-4031 (2019).

12 Choe, S. S., Huh, J. Y., Hwang, I. J., Kim, J. I., Kim, J. B. Adipose Tissue Remodeling: Its Role in Energy Metabolism and Metabolic Disorders. *Frontiers in Endocrinology (Lausanne).* **7**, 30 (2016).

13 Hepler, C., Vishvanath, L., Gupta, R. K. Sorting out adipocyte precursors and their role in physiology and disease. *Genes and Development.* **31** (2), 127-140 (2017).

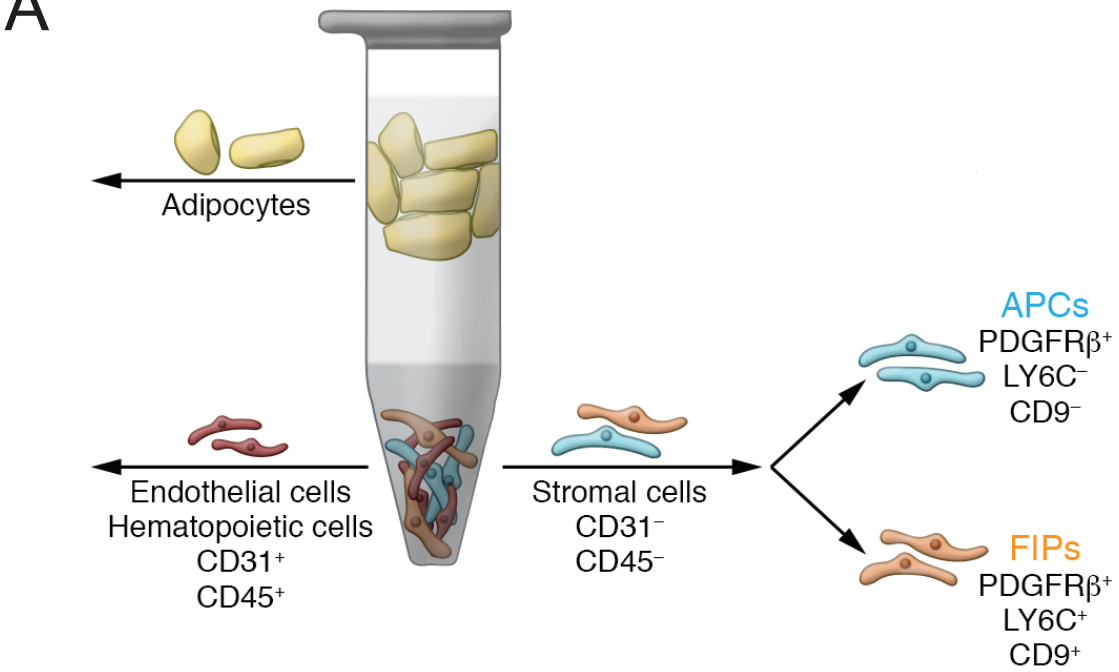
14 Burl, R. B. et al. Deconstructing Adipogenesis Induced by beta3-Adrenergic Receptor Activation with Single-Cell Expression Profiling. *Cell Metabolism.* **28** (2), 300-309 (2018).

15 Hepler, C. et al. Identification of functionally distinct fibro-inflammatory and adipogenic stromal subpopulations in visceral adipose tissue of adult mice. *Elife.* **7**, e39636, (2018).

16 Merrick, D. et al. Identification of a mesenchymal progenitor cell hierarchy in adipose tissue. *Science.* **364** (6438), aav2501 (2019).
17 Schwalie, P. C. et al. A stromal cell population that inhibits adipogenesis in mammalian fat depots. *Nature.* **559** (7712), 103-108 (2018).
18 Bagchi, D. P., MacDougald, O. A. Identification and Dissection of Diverse Mouse Adipose Depots. *Journal of Visualized Experiments.* (149), e59499 (2019).
19 Jeffery, E., Church, C. D., Holtrup, B., Colman, L., Rodeheffer, M. S. Rapid depot-specific activation of adipocyte precursor cells at the onset of obesity. *Nature Cell Biology.* **17** (4), 376-385 (2015).
20 Kim, S. M. et al. Loss of white adipose hyperplastic potential is associated with enhanced susceptibility to insulin resistance. *Cell Metabolism.* **20** (6), 1049-1058 (2014).
21 Vishvanath, L. et al. Pdgfrbeta+ Mural Preadipocytes Contribute to Adipocyte Hyperplasia Induced by High-Fat-Diet Feeding and Prolonged Cold Exposure in Adult Mice. *Cell Metabolism.* **23** (2), 350-359 (2016).
22 Wang, Q. A., Tao, C., Gupta, R. K., Scherer, P. E. Tracking adipogenesis during white adipose tissue development, expansion and regeneration. *Nature Medicine.* **19** (10), 1338-1344 (2013).
23 Gao, Z., Daquinag, A. C., Su, F., Snyder, B., Kolonin, M. G. PDGFRalpha/PDGFRbeta signaling balance modulates progenitor cell differentiation into white and beige adipocytes. *Development.* **145** (1), e155861 (2018).
24 Rodeheffer, M. S., Birsoy, K., Friedman, J. M. Identification of white adipocyte progenitor cells in vivo. *Cell.* **135** (2), 240-249 (2008).
25 Church, C. D., Berry, R., Rodeheffer, M. S. Isolation and study of adipocyte precursors. *Methods Enzymol.* **537**, 31-46 (2014).
26 Buffolo, M. et al. Identification of a Paracrine Signaling Mechanism Linking CD34(high) Progenitors to the Regulation of Visceral Fat Expansion and Remodeling. *Cell Reports.* **29** (2), 270-282, (2019).
27 Shao, M. et al. De novo adipocyte differentiation from Pdgfrbeta(+) preadipocytes protects against pathologic visceral adipose expansion in obesity. *Nature Communications.* **9** (1), 890 (2018).
28 Lee, P. Y., Wang, J. X., Parisini, E., Dascher, C. C., Nigrovic, P. A. Ly6 family proteins in neutrophil biology. *Journal of Leukocyte Biology.* **94** (4), 585-594 (2013).
29 Vijay, J. et al. Single-cell analysis of human adipose tissue identifies depot and disease specific cell types. *Nature Metabolism.* **2** (1), 97-109 (2020).

Figure 1





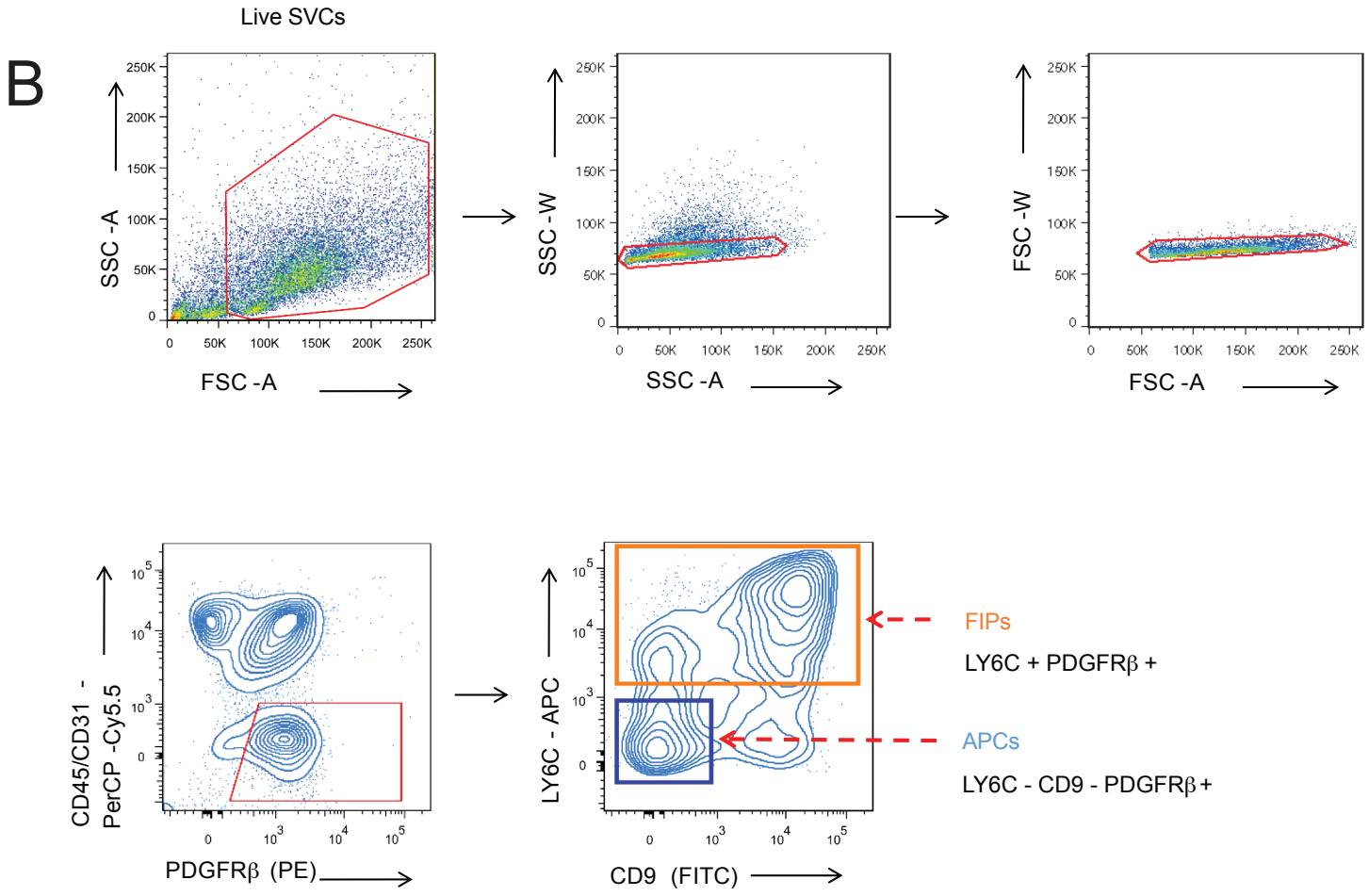
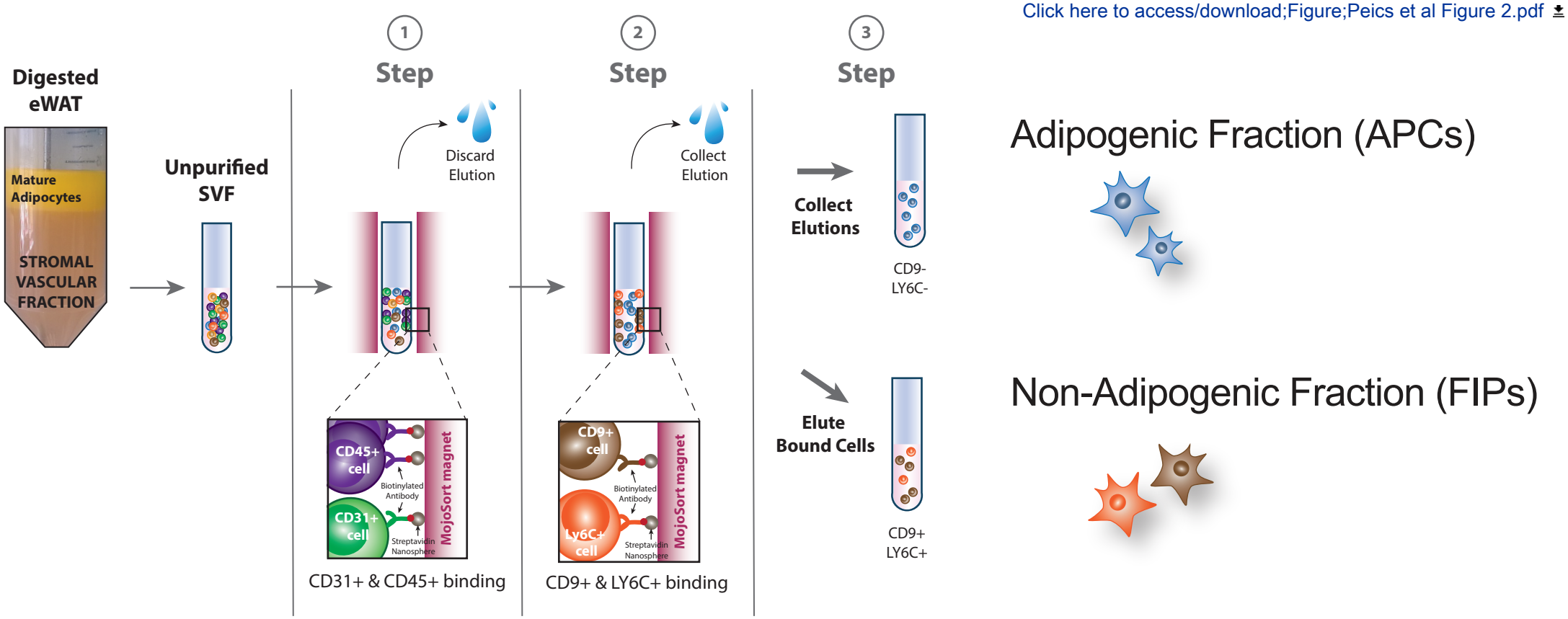




Figure 1

Figure 2



A




B

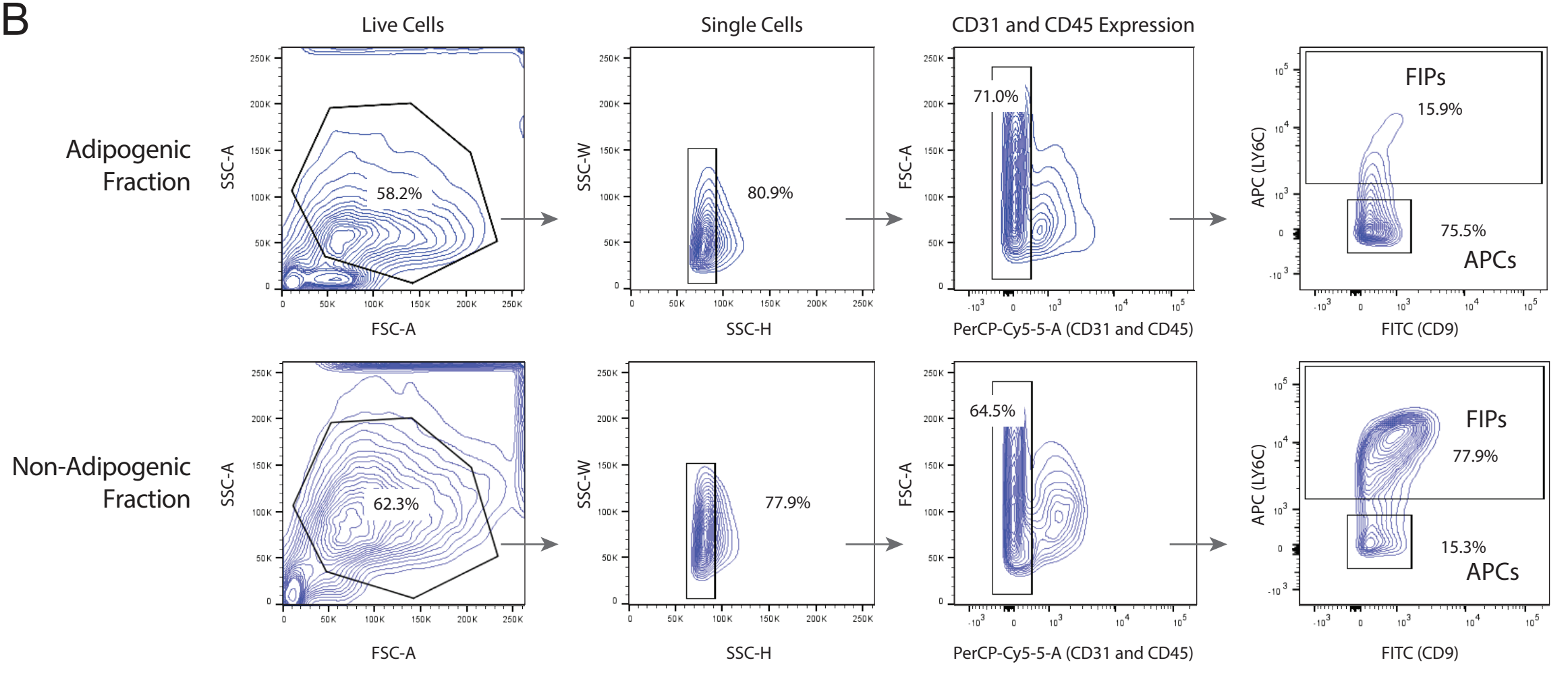


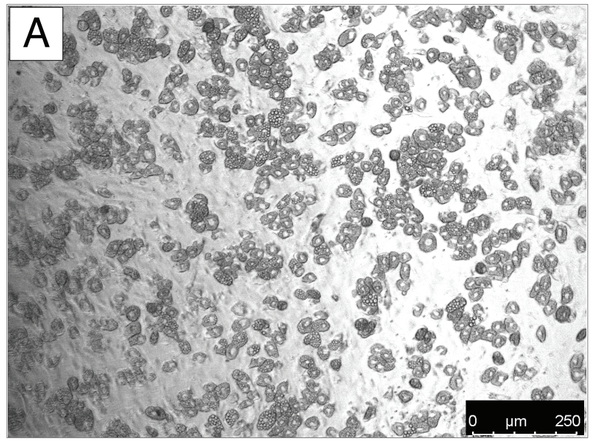

Figure 2

Figure 3

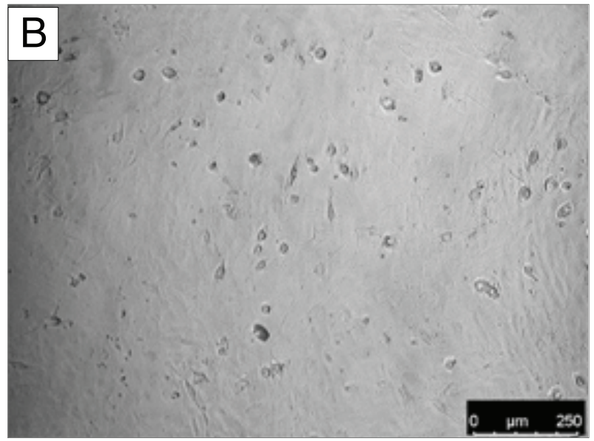


## Immunomagnetic Cell Separation

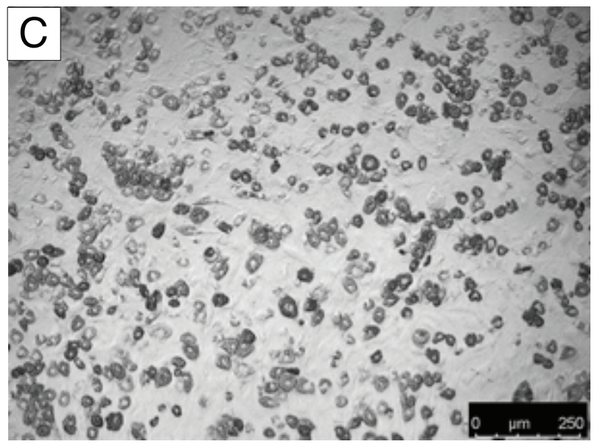
## FACS



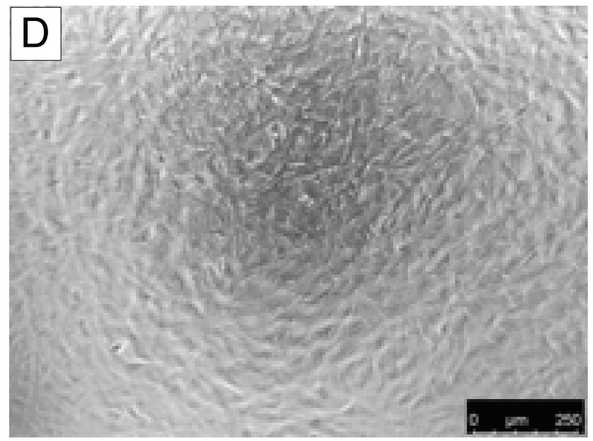

APCs



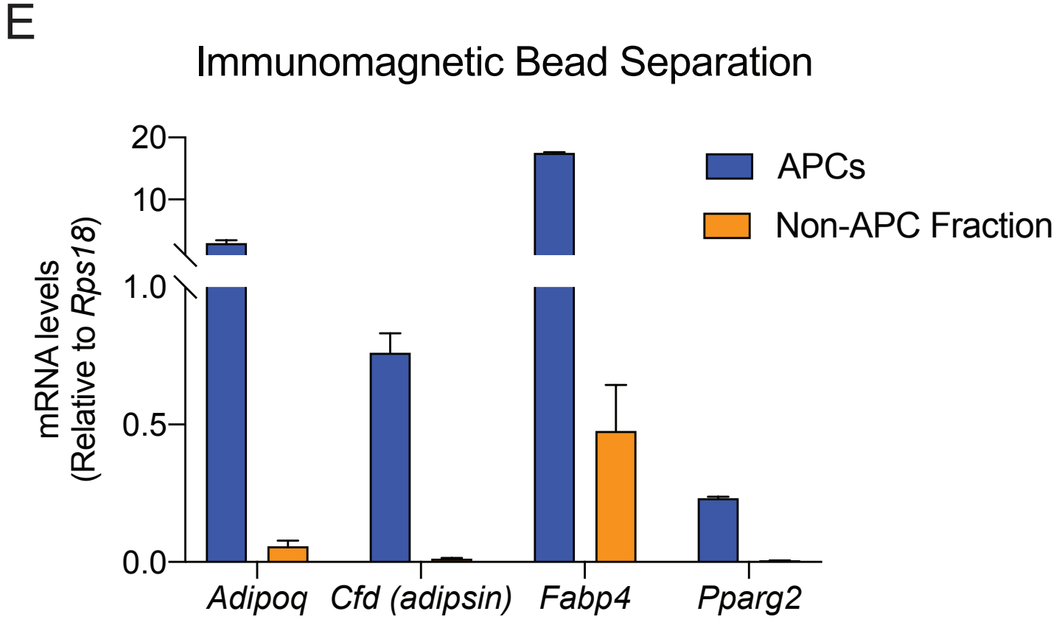

Non-APC Fraction




APCs




FIPs



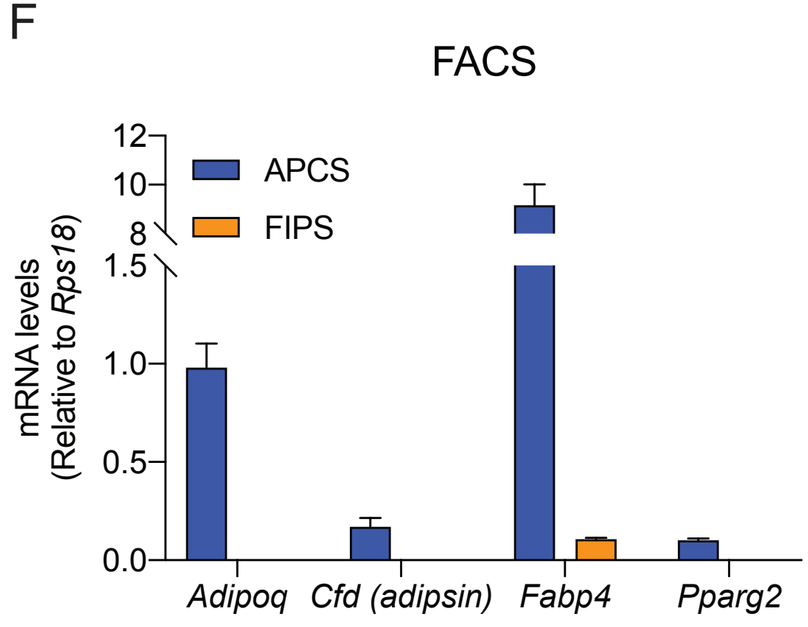




Figure 3

Table 1: qPCR primers sequences used

| FIPs | Enriched Genes (vs. APCs) |
|---|---|
| | *Ly6c1* |
| | *CD9* |
| | *Nov* |
| | *Efhd1* |
| | *Stmn4* |
| | *Dact2* |
| | *Il33* |
| | *Ccl2* |
| | *Tgfb2* |
| | *Fn1* |
| | *DPP4* |
| | *Thy1* |

| APCs | Enriched Genes (vs. FIPs) |
|---|---|
| | *Agt* |
| | *Cxcl14* |
| | *Mmd2* |
| | *Pde11a* |
| | *Lrn1* |
| | *Pparg* |
| | *Fabp4* |
| | *Lpl* |
| | *Cd36* |

d to validate the isolation of FIPs and APC

| Forward Primer 5’-3’ |
| --- |
| ACTGTGCCTGCAACCTTGTCT |
| GCGGGAAACACTCAAAGCCAT |
| GTTCCAAGAGCTGTGGAATGG |
| GGCCGCTCTAAGGTCTTCAAT |
| ACCTGAACTGGTGCGTCATCT |
| AGCCCCCTAAAGGAAGAAACC |
| ATTTCCCCGGCAAAGTTCAG |
| CCACAACCACCTCAAGCACTTC |
| GGTGTTGTTCCACAGGGGTTA |
| GAGAGCACACCCGTTTTCATC |
| TGGTGGATGCTGGTGTGGATT |
| TCTTCTTTCCCTTGCCCCTCTG |

| Forward Primer 5’-3’ |
| --- |
| GTTCTGGGCAAAACTCAGTGC |
| TGGACGGGTCCAAGTGTAAGT |
| ATCTGGGAGCTGATGACAGGA |
| CGAGCTTGTCAGGAAAGGAGA |
| CAACATGGGAGAGCTGGTTTC |
| GCATGGTGCCTTCGCTGA |
| ACTGGGCGTGGAATTCGATGA |
| CATCGAGAGAGGATCCGAGTGAA |
| GAGTTGGCGAGAAAACCAGTG |

:s

| Reverse Primer 5’-3’ |
|---|
| GGCCACAAGAAGAATGAGCAC |
| AAAGCTGTTTCTTGGGGCAGG |
| CTCTTGTTCACAAGGCCGAAC |
| GTCAATAAAGCCGTCCCTTCC |
| CTTGGGAGGGAGGCATTAAAC |
| GGTCCTTGGCCACAGTCATTA |
| AACGGAGTCTCATGCAGTAGA |
| AAGGCATCACAGTCCGAGTCAC |
| CGGTCCTTCAGATCCTCCTTT |
| GGGTCCACATGATGGTGACTT |
| AAGGGGCCTCTCTTCTCTTCCT |
| AGGTTGCAAGACTCTCGCTGT |

| Reverse Primer 5’-3’ |
|---|
| GAGGCTCTGCTGCTCATCATT |
| TCCTCGCAGTGTGGGTACTTT |
| AGTGGGTACCAGCACCAAATG |
| TTCAGCCACCTGTCTGGAGAT |
| GCACACTACGGAAAGCCAAAC |
| TGGCATCTCTGTGTCAACCATG |
| ACCAGCTTGTCACCATCTCGT |
| TGCTGAGTCCTTTCCCTTCTG |
| GAGAATGCCTCCAAACACAGC |

| Name of Material/Equipment | Company | Catalog Number | Concentration | Species |
|---|---|---|---|---|
| **Mechanical Tissue Preparation and SVF Isolation** | | | | |
| 40 and 100 µm cell strainers | Fisher Scientific | 352340/352360 | | |
| 1X Phosphate buffered saline (PBS) | Fisher Scientific | 21040CV | | |
| 5ml polypropylene tubes | Fisher Scientific | 352053 | | |
| **Digestion Buffer (for 10mL)** | | | | |
| 10 ml HBSS | Sigma | H8264 | | |
| 10 mg Collagenase D (1 mg/ml final cc.) | Roche | 11088882001 | | |
| 0.15 g BSA (1.5 % final cc.) | Fisher Scientific | BP1605-100 | | |
| **Immunomagnetic separation of APCs and non-APCs** | | | | |
| 5X MojoSort Buffer (MS buffer) | BioLegend | 480017 | | |
| 5 ml MojoSort Magnet (MS magnet) | BioLegend | 480019 | | |
| 100 µL MojoSort Streptavidin Nanobeads | BioLegend | 480015 | | |
| **Purity Check and FACS** | | | | |
| 10X Red Blood Cell Lysis Buffer | eBioscience | 00-4300-54 | | |
| Fc block (Mouse CD16/CD32) | eBioscience | 553141 | | |
| **Antibodies** | | | | |
| Biotin CD45 | BioLegend | 103103 | ≤ 0.25 µg per 10^6 cells | Mouse |
| Biotin CD31 | BioLegend | 102503 | ≤ 0.25 µg per 10^6 cells | Mouse |
| Biotin CD9 | BioLegend | 124803 | ≤ 0.25 µg per 10^6 cells | Mouse |
| Biotin LY6C | BioLegend | 128003 | ≤ 0.25 µg per 10^6 cells | Mouse |
| CD31-PerCP/Cy5.5 | BioLegend | 102419 | Dilution 1:400 | Mouse |
| CD45-PerCP/Cy5.5 | BioLegend | 103131 | Dilution 1:400 | Mouse |
| CD140b PDGFRβ-PE | BioLegend | 136006 | Dilution 1:50 | Mouse |
| LY6C-APC | BioLegend | 128016 | Dilution 1:400 | Mouse |
| CD9-FITC | BioLegend | 124808 | Dilution 1:400 | Mouse |
| **Cell Culture and Differentiation** | | | | |
| **Gonadal APC Culture media (for 500mL)** | | | | |

| | | |
|---|---|---|
| 288 mL DMEM with 1 g/L glucose | Corning | 10-014-CV |
| 192 mL MCDB201 | Sigma | M6770 |
| 10 mL Fetal bovine serum (FBS)** lot#14E024 | Sigma | 12303C |
| 5 mL 100% ITS premix | BD Bioscience | 354352 |
| 5 mL 10 mM L-ascorbic acid-2-2phosphate | Sigma | A8960-5G |
| 50 µL 100 g/ml FGF-basic | R&D systems | 3139-FB-025/CF |
| 5 mL Pen/Strep | Corning | 30-001-CI |
| 500 µL Gentamycin | Gibco | 15750-060 |

**NOTE: The adipogenic capacity of primary APCs can vary from lot to lot of commercial FBS. Multiple lots/sources of FBS should be tested.

| Clone | Comments/Description |
| --- | --- |
| 30-F11 | |
| MEC13.3 | |
| MZ3 | |
| HK1.4 | |
| 390 | |
| 30-F11 | |
| APB5 | |
| HK1.4 | |
| MZ3 | |

**Point by Point Response to Editorial and Reviewer Critiques**

**Editorial comments:**
***Changes to be made by the Author(s):***
***1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.***

Thank you. We have done our best to ensure that there are no spelling or grammar issues.

***2. Please format the manuscript as: paragraph Indentation: 0 for both left and right and special: none, Line spacings: single. Please include a single line space between each step, substep and note in the protocol section. Please use Calibri 12 points***

Done.

***3. Please define all abbreviations during the first-time use.***

Done.

***4. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., "Do this," "Ensure that," etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as "could be," "should be," and "would be" throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a "Note."***

Done.

***5. Please adjust the numbering of the Protocol to follow the JoVE Instructions for Authors. For example, 1 should be followed by 1.1 and then 1.1.1 and 1.1.2 if necessary. Please refrain from using bullets, alphabets, or dashes.***

Done.

***6. The Protocol should contain only action items that direct the reader to do something.***

Done.

***7. Please ensure that individual steps of the protocol should only contain 2-3 actions sentences per step.***

Done.

***8. Please ensure you answer the "how" question, i.e., how is the step performed?***

Done.

***9. There is a 10-page limit for the Protocol, but there is a 2.75-page limit for filmable content. Please highlight 2.75 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol.***

Done.

***10. Please remove the embedded Table from the manuscript. All tables should be uploaded separately to your Editorial Manager account in the form of an .xls or .xlsx file. Each table must be accompanied by a title and a description after the Representative Results of the manuscript text.***

Done.

***11. Please revise the table of the essential supplies, reagents, and equipment. The table should include the name, company, and catalog number of all relevant materials in separate columns in an xls/xlsx file.***

Done.

***12. Please obtain explicit copyright permission to reuse any figures from a previous publication. Explicit permission can be expressed in the form of a letter from the editor or a link to the editorial policy that allows re-prints. Please upload this information as a .doc or .docx file to your Editorial Manager account. The Figure must be cited appropriately in the Figure Legend, i.e. "This figure has been modified from [citation]."***

Done.

***13. As we are a methods journal, please ensure that the Discussion explicitly cover the following in detail in 3-6 paragraphs with citations:***
***a) Critical steps within the protocol***
***b) Any modifications and troubleshooting of the technique***
***c) Any limitations of the technique***
***d) The significance with respect to existing methods***
***e) Any future applications of the technique***
***14. Please do not abbreviate the journal titles in the reference section.***

Done.

**Reviewers' comments:**
**Reviewer #1:**
***Manuscript Summary:***
***Leaders in this field are going to share with other investigators what they've discovered about FACs sorting stromal vascular cells.***

***Major Concerns:***
***none***

***Minor Concerns:***
***none***

We thank the reviewer for taking the time to review our manuscript.

**Reviewer #2:**

***The stromal-vascular fraction of white adipose tissue is highly heterogeneous. The authors here describe a method for the isolation of functionally distinct adipose progenitor subpopulations from murine intra-abdominal adipose depots, i.e. fibro-inflammatory progenitors and adipocyte precursor cells. These two cell populations can be isolated by FACS or biotinylated antibody-based immunomagnetic bead technology. The manuscript is well prepared and the protocol is described in great detail. I have no doubt that these methods will greatly help understand better the molecular mechanisms regulating adipogenesis and intra abdominal adipose tissue remodeling in health and disease. I only have minor suggestions to make:***

We thank the reviewer for taking the time to carefully review the manuscript.

***1) The results show examples of APC enriched genes. It would be helpful to show FIP specific/enriched genes as well***

The data presented in the current paper show the expression of adipocyte-selective genes/markers within cultured subpopulations following adipogenesis. These data are meant to illustrate the difference in adipogenic capacity. We refer readers to the original research article about these cell populations for a detailed analysis of FIPs/APCs gene expression profiles. To help investigators, we have now incorporated a table in the manuscript with a list of genes enriched in FIPs and APCs along with the respective primer sequences. These genes can be used as markers of the sorted populations.

***2) The authors should discuss the strengths and weaknesses of the 2 methods they describe. Would the authors recommend one or the other depending on different downstream applications? Are there big differences in terms of cost, time, quality/purity?***

This is an important point. We have now revised the discussion section to better discuss this issue.

***3) At what temperature are performed the centrifugation steps?***

We regret the omission of this important detail. Centrifugation was performed at 4C. We have added the centrifugation temperature in the protocol.

***4) The authors use 6-8 week old mice. The authors should discuss if mice of different ages can be used, or if the protocol works better in 6-8 week old mice***

This is a great question. We recommend using younger mice that are about 6-8 weeks old for better yield of APCs and FIPs. We have observed that the frequency of PDGFRβ+ APCs decrease as animals get older. Isolation of APCs from older mice may yield fewer cells. We have now added this note to the protocol and thank the reviewer for pointing this out.

***5) Step1.2: "combine 2-4 fat depots": Is it different depots from one mouse or the same depot from several mice? Is there any downside to pool tissues from different mice?***

We use gonadal white adipose tissue in this protocol for isolation of FIPs and APCs. We isolate two depots from one mouse (left and right) and combine up to four depots from two mice in one tube of 10ml digestion buffer. There is no downside of pooling tissue as long as the mice are of similar age and same sex. We do not recommend combining more than 4 depots in 10ml digestion buffer to avoid cell saturation/clogging of filters. However, several mice and multiple tubes of digestion buffer may be used and cells may be combined at the end of the isolation for further assays. We have now added this note to the protocol.

***6) Are these methods specific to mice? The authors should discuss which species the techniques can be used with***

This is a good question. At present, we can only conclude that these isolation methods and in vitro culture conditions discussed here can be used for murine intraabdominal adipose depots only. We have not tested this protocol with rat adipose tissue. Importantly, humans do not have an apparent LY6C ortholog; therefore, this particular separation strategy cannot be used with human WAT. We have now added this point to the discussion section of the manuscript and thank the reviewer for raising this issue.

**Reviewer #3:**

GENERAL COMMENTS

The article by Peics and collaborators describes two methods, one using fluorescence-activated cell sorting and the other using immunomagnetic beads, allowing isolation of two distinct cell populations from the stromal-vascular cell fraction of adipose tissue. Taking advantage of the specific cell-surface antigen signature, the authors were able to isolate fibro-inflammatory precursors (LY6C+, PDGFRb+, CD45-, CD31-) and adipocyte precursors (CD45-, CD31- , LY6C-, PDGFRb+, CD9-). The methodology used for this protocol description is not novel. What is original is its application to the separation of cell fractions from adipose tissue. However, more proof would be required to ascertain that the phenotype of the cells isolated is consistent with the label claimed by the Authors. Specific comments are provided below.

***MAJOR COMMENTS***

***1- As mentioned above, more phenotypic characterization would greatly reinforce the relevance of the cell type separation proposed here. Additional experiments would be required on APC cells.***

***2- Bright-field microscope pictures suggest that APC accumulate intracellular lipids. However, cells are not stained and no quantification is provided. We suggest Oil Red O staining in both APC and FIP with red-pixel quantification.***

We thank the reviewer for taking the time to review the manuscript and to offer constructive feedback. The focus of this manuscript is to provide a detailed method for separation of two stromal subpopulations that have been described in our prior publication. A detailed characterization for the functional properties of these cells has already been performed, peer-reviewed, and published in Hepler et al. *Elife* 2018. Given the scope of a methods paper for JoVE, we refer to the published literature regarding the characterization of these cell populations. Of note, our prior publication includes several functional assays of adipogenesis [in vitro (with ORO staining) and in vivo] and pro-inflammatory responses. In the revised manuscript, we direct readers to this prior publication for further insight into the functional properties of these cells.

***3- APC seem to differentiate spontaneously. However, this might be due to the presence of insulin in the medium (ITS premix). Is the same process present when ITS is not added? What kind of medium was used on FIP in Figure 3B and 3D? To allow comparison, both cells should have received APC medium.***

This is a great point. There is indeed a significant amount of insulin in the ITS premix. We have amended the discussion to clarify this point. Importantly, both APCs and FIPs did receive the same culture medium. We have amended the manuscript (figure legend and discussion section) to make this clearer.

***4- How long were the cells cultivated for when PCR analyses were performed?***

Cells were harvested for qPCR analysis after APCs were fully differentiated. APCs begin to undergo spontaneous differentiation as they approach confluence and take about 7-10 days to fully differentiate. FIPs were harvested at the same time as APCs. We have amended the protocol to make this clearer.

***5- Similar to APC, gene expression of FIP could be investigated. Genes that could be of interest are: TNF-alpha, TGF-beta, collagen and/or metalloproteases, among others.***

The data presented in the current paper show the expression of adipocyte-selective genes/markers within cultured subpopulations following adipogenesis. These data are meant to illustrate the difference in adipogenic capacity. We refer readers to the original research article about these cell populations for a detailed analysis of FIPs/APCs gene expression profiles. To help investigators, we have now incorporated a table in the manuscript with a list of genes enriched in FIPs and APCs along with the respective primer sequences. These genes can be used as markers of the sorted populations.

***6- Additional experiments could include localization of FIP and APC in whole adipose tissue by immuofluorescence staining.***

This is an important question; however, it is outside of the scope of this methods paper which is solely dedicated to presenting strategies to isolate the cells. We are working on developing strategies to localize the cells using commercially available antibodies. This will be included in a future publication.

***7- Line 67: Excess caloric intake is not the sole factor inducing pathological remodeling of adipose tissue. Indeed, many studies show that the metabolic and functional characteristics of what is defined as 'metabolically healthy obesity' are likely to deteriorate with time.***

Good point. We have amended this sentence to read, “Moreover, insulin resistance in obesity is associated with pathologic remodeling of WAT.”

***8- Overall, the paper lacks many references to support some statements. For example, line 78 when describing a microenvironment that influences AT expansion; line 95 when describing FIP; line 100 when stating that APC readily differentiate into mature adipocytes.***

We thank the reviewer for pointing out these omissions. We have added the appropriate references where indicated.

MINOR COMMENTS

***1- Among papers that identify various cell populations in the stromal vascular fraction by single cell sequencing, it may of interest to cite the recently published paper by Vijay and collaborators (PMID: 32066997). These authors characterized and compared the stroma-vascular fraction of obese patients with type 2 diabetes vs. those without type 2 diabetes. That study could add relevance of the method proposed here.***

Great point. We now make reference to this paper in the revised Discussion as we refer to the point below. We thank the reviewer for this suggestion.

***2- Do the two cell populations investigated here (FIP vs. APC) exist also in human stromal-vascular fraction? In what measure are they different or similar to the ones found in humans?***

This is a great question. Based on our unpublished work and our cursory analysis of published datasets, we think that cells reminiscent of the FIPs and APCs we describe are indeed present in human tissue. However, we are not yet able to draw any conclusions until we isolate the putative populations and test them functionally. This is part of an on-going effort in the lab. Importantly, humans do not have a LY6C ortholog; therefore, our

sorting strategy cannot be used to isolate APCs from human WAT. We now make this latter point clear in the manuscript.

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| **Title, description or numeric reference of the portion(s)** | Figure 2B | **Title of the article/chapter the portion is from** | Contribution of adipogenesis to healthy adipose tissue expansion in obesity |
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