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TITLE:

Robust Differentiation of Human iPSCs into a Pure Population of Adipocytes to Study Adipocyte-Associated Disorders

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KEYWORDS:

induced pluripotent stem cells, mesenchymal stem cells, adipocyte differentiation, cell sorting

SUMMARY:

The protocol allows the generation of a pure adipocyte population from induced pluripotent stem cells (iPSCs). Retinoic acid is used to differentiate iPSCs into mesenchymal stem cells (MSCs) which are used for producing adipocytes. Then, a sorting approach based on Nile red staining is used to obtain pure adipocytes.

ABSTRACT:

Recent advances in induced pluripotent stem cell (iPSC) technology have allowed the generation of different cell types, including adipocytes. However, the current differentiation methods have low efficiency and do not produce a homogenous population of adipocytes. Here, we circumvent this problem by using an all-trans retinoic-based method to produce mesenchymal stem cells (MSCs) in high yield. By regulating pathways governing cell proliferation, survival, and adhesion, our differentiation strategy allows the efficient generation of embryonic bodies (EBs) that differentiate into a pure population of multipotent MSCs. The high number of MSCs generated by this method provides an ideal source for generating adipocytes. However, sample heterogeneity resulting from adipocyte differentiation remains a challenge. Therefore, we used a Nile red-based method for purifying lipid-bearing mature adipocytes using FACS. This sorting strategy allowed us to establish a reliable way to model adipocyte-associated metabolic disorders using a pool of adipocytes with reduced sample heterogeneity and enhanced cell functionality.

INTRODUCTION:

Mesenchymal stem cells (MSCs) act as an effective transitory resource for producing cells of mesodermal origin like adipocytes, osteocytes, and chondrocytes, which could be further used for modeling their respective genetic disorders. However, previous approaches relied on attaining these MSCs from adult tissues[1], which imposed the challenge of obtaining them in high numbers from the donors, and the limitation of keeping them functionally viable in suboptimal *in vitro* culture conditions[1,2]. These obstacles have produced a great demand of having a protocol for generating MSCs *in vitro*. Human induced pluripotent stem cells (iPSCs) can be used as a valuable source of MSCs, exhibiting MSC characteristics[3–5]. iPSCs-derived MSCs can be used as a therapeutic option in several diseases. Also, the ability of iPSCs-derived MSCs to generate adipocytes, makes them a valuable *in vitro* human model to study human adipogenesis, obesity, and adipocyte-associated disorders.

Current differentiation protocols of adipocytes can be classified into two groups, with one involving differentiation of adipocytes using chemical or protein-based cocktails giving a resultant yield of 30%–60%[6–9], while the other involving genetic manipulation for robust induction of key transcription factors governing adipocytes development to give a yield of 80%–90%[10,11]. However, genetic manipulation doesn't recapitulate the natural process of adipocyte differentiation, and often masks the subtle paradigms arriving during adipogenesis, making it ineffective for disease modeling purposes[12,13]. Therefore, we present a way to sort chemically derived mature adipocytes from immature ones by fluorescently tagging lipid-bearing adipocytes using Nile red.

Here we present a protocol involving transient incubation of iPSCs derived embryoid bodies (EBs) with all-*trans* retinoic acid to produce a high number of rapidly proliferating MSCs, which could be further used for generating adipocytes[14]. We also present a way to sort chemically derived mature adipocytes from the heterogeneous differentiation pool by fluorescently tagging their lipid droplets using a lipophilic dye; Nile red. This would allow the generation of a pure population of mature adipocytes with enhanced functionality to accurately model adipocyte-associated metabolic disorders.

PROTOCOL:

The study has been approved by the appropriate institutional research ethics committee and performed following the ethical standards as laid down in the 1964 Declaration of Helsinki and its later amendments or comparable ethical standards. The protocol was approved by the Institutional Review Board (IRB) of HMC (no. 16260/16) and QBRI (no. 2016-003). This work is also optimized for hESCs such as H1 and H9. Blood samples were obtained from healthy individuals with full informed consent. The iPSCs are generated from peripheral blood mononuclear cells (PBMCs) of healthy individuals.

1. Culturing and maintaining iPSCs

1.1. Prepare basement membrane matrix-coated plates by reconstituting coating matrix in knockout-DMEM at a ratio of 1:80 and store at 4 °C.

1.2. Prepare iPSCs culture media by adding 50 mL of 10x stem cell supplement media to 500 mL of stem cell basal media, along with 5 mL of 100x penicillin-streptomycin (P/S) and store at 4 °C for short term or at -20 °C for long term use.

1.3. Line the plates with coating matrix—1 mL for a 6-well plate, 500 µL for a 12-well plate, 250 µL for a 24-well plate—and incubate the plate at 37 °C for 1–2 h.

1.4. Remove an aliquot of iPSCs culture media and pre-warm at room temperature before use.

1.5. Thaw a vial of iPSCs (ESCs or iPSCs) in a 37 °C water bath and transfer to a 15 mL conical tube containing 2–3 mL of culture media.

1.6. Centrifuge the tube at 120 x *g* for 4 min at room temperature (RT—23 °C).

1.7. Remove the supernatant and add 2 mL of fresh culture media supplemented with 10 µM ROCK inhibitor (Y-27632). Plate the cells in one well of a matrix-coated 6-well plate and place the plate at 37 °C.

1.8. After 24 h, remove the media and replace it with fresh culture media.

1.9. Change the media every day until the cells reach 80%–90% confluency.

1.10. Upon reaching confluency, passage cells by following the steps outlined below.

1.10.1. Remove the media and wash the cells with Dulbecco's phosphate-buffered saline (DPBS).

1.10.2. Add iPSCs dissociation reagent (see **Table of Materials**)—500 µL for a well of a 6-well plate, 250 µL for a well of a 24-well plate—and incubate for 1 min at 37 °C.

1.10.3. Remove the dissociation reagent and incubate the cells dry for 1 min at 37 °C.

1.10.4. Collect the cells using culture media—1 mL for a well of a 6-well plate and 250 µL for a well of a 24-well plate—in a 15 mL conical tube and centrifuge at 120 x *g* for 4 min.

1.10.5. Resuspend cells in culture media—2 mL for a well of a 6-well plate and 500 µL for a well of a 24-well plate—supplemented with 10 µM ROCK inhibitor and plate them on fresh matrix coated plates at 40% confluency.

2. Differentiation of iPSC into MSCs

2.1. Prepare MSC differentiation media by adding 15% fetal bovine serum (FBS) and 1% P/S to low glucose DMEM + pyruvate and store at 4 °C.

2.2. Upon reaching 80% confluency, use iPSCs for embryoid body (EB) formation following the steps outlined below.

2.2.1. Wash the cells with DPBS and incubate them with dissociation medium/EDTA—500 µL for a well of a 6-well plate, 250 µL for a well of a 24-well plate.

2.2.2. Incubate at 37 °C for 1 min, aspirate the dissociation reagent and keep the cells at 37 °C for an additional 1 min. To start MSC differentiation, ~10–12 x 10^6 cells are required.

2.2.3. Collect the cells in a 15 mL conical tube using culture media. Make sure to be very gentle while collecting to prevent cells from getting single and allow EB formation. Centrifuge the cells at 120 x *g* for 4 min.

2.2.4. Resuspend the cells in 3 mL of MSC differentiation media containing 10 µM ROCK inhibitor.

2.2.5. Mix and distribute 0.5 mL/well in a 24-well ultra-low attachment plate.

NOTE: Usage of ultra-low attachment plate would encourage cell aggregation into EBs rather than their attachment on the surface.

2.2.6. Place the plate in the incubator at 37 °C.

2.3. After 24 h, induce the attained EBs with high retinoic acid (RA) treatment by following the steps outlined below.

2.3.1. Add 10 µM RA to 3 mL of MSC differentiation media. Collect EBs in a 15 mL tube and allow them to settle down for 15 min.

2.3.2. Remove the supernatant from EBs and add MSC differentiation media supplemented with 10 µM RA.

2.3.3. Resuspend gently and distribute 0.5 mL/well in the same 24-well ultra-low attachment plate.

2.3.4. Place the plate in the incubator at 37 °C. Do not disturb EBs for the next 48 h.

2.3.5. After 48 h, collect EBs in a 15 mL tube and allow them to settle down for 15 min.

2.3.6. Remove the supernatant from EBs and add MSC differentiation media supplemented with 0.1 µM RA.

2.3.7. Resuspend gently and distribute 0.5 mL/well in the same 24-well ultra-low attachment plate.

2.3.8. Place the plate in the incubator at 37 °C. Do not disturb EBs for the next 48 h.

2.4. Remove the RA added to the cells by following the steps outlined below.

2.4.1. After 48 h of the last RA treatment, collect the EBs and allow them to settle down for 15 min.

2.4.2. Remove the supernatant and add DMEM low glucose media without cytokines.

2.4.3. Resuspend gently and distribute 0.5 mL/well in a 24-well ultra-low attachment plate. Place the plate in the incubator at 37 °C.

2.5. Plate the iPSCs-derived EBs by following the steps outlined below.

2.5.1. After 48 h from the previous step (step 2.4), collect the EBs in a 15 mL tube and allow them to settle down for 15 min.

2.5.2 Remove supernatant and resuspend in 2 mL of fresh MSC differentiation media.

2.5.3 Transfer to two wells of a basement membrane matrix-coated 6-well plate.

2.5.4 Change the media every other day for additional 5 days.

2.5.5 After 5 days, remove the spent media and replace it with fresh MSC differentiation media containing 2.5 ng/mL of basic fibroblast growth factor (bFGF).

2.6 Passage the plated EBs when they reach 80%–90% confluency, by following the steps outlined below.

2.6.1. Wash the cells with DPBS, add trypsin-EDTA—500 µL for a well of a 6-well plate—and incubate the cells at 37 °C for 3 min.

2.6.2. Collect the cells using MSC differentiation media in a 15 mL conical tube and spin at 750 x *g* for 4 min.

2.6.3. Resuspend in MSC differentiation media with 2.5 ng/mL of bFGF and plate the cells on basement membrane matrix-coated plates at a ratio of 1:3.

2.6.4. Repeat the passage when the cells reach 70%–80% confluency. It is expected to gain 3–6 million cells by 2–3 passages.

3. Flow cytometry analysis of iPSCs-derived MSCs

NOTE: Upon undergoing 2–3 passages, the cells should be accessed for the efficiency of MSC differentiation. Differentiation will be considered successful if the cells express MSC differentiation markers—CD44, CD73, CD90, and CD105 at more than 90% efficiency, and do not express high levels of hematopoietic markers—CD14, CD19, CD34, and CD45. The efficiency of these markers can be accessed by following the steps below.

3.1. Passage the cells using the steps outlined above (step 2.6) and attain 1 x 10^5 cells in one well of a v-bottom 96-well plate.

3.2. Centrifuge the plate at 750 x *g* for 4 min at 4 °C.

3.3. Resuspend 1 x 10^5 cells in 100 µL of cold DPBS with 1 µL of conjugated antibody (Ab) (see **Table of Materials**) and incubate at 4 °C for 30–40 min preventing exposure to light.

3.4. Resuspend another 1 x 10^5 cells in 100 µL of cold DPBS with the respective isotype control of the conjugated Ab at a concentration of 1:100) and incubate at 4 °C for 30–40 min preventing exposure to light.

3.5. Following incubation, centrifuge the plate at 750 x *g* for 4 min at 4 °C. Discard the supernatant by shaking the plate over the sink.

3.6. Resuspend the cells in 100 µL of cold DPBS.

3.7. Centrifuge the cells at 750 x *g* for 4 min at 4 °C. Discard the supernatant.

3.8. Resuspend the cells in 200 µL of cold DPBS and collect in dark, cold 1.5 mL microcentrifuge tubes and keep them on ice until analyzed by fluorescence-activated cell sorting (FACS).

3.9. For FACS analysis, distribute the cells using side scattered (SSC-A) versus forward scattered (FSC-A) to exclude the debris. Further, distribute the gated cells using forward scattered height (FSC-H) versus forward scattered area (FSC-A) to distinguish singlets from doublets from the live cell population.

NOTE: Cells were gated relative to the shift of isotype control for every marker, and a minimum of 10,000 gated events from every stained sample was used for analysis.

4. Differentiation of MSCs into adipocytes

4.1. Prepare adipocyte differentiation basal media by adding 10% knockout serum replacement (KOSR), 1% Glutamine, 1% P/S, 4.5 ng/µL of glucose to minimum essential media (MEM)-alpha and store at 4 °C.

4.2. Allow MSCs to reach above 90% confluency. Continue culturing them for another 48 h to allow them to undergo a period of growth arrest.

4.3. Prepare complete adipocyte differentiation media by adding 100 µg/mL of 3-Isobutyl-1-methylxanthine (IBMX), 1 µM of dexamethasone, 0.2 U/mL of insulin, 100 µM of indomethacin, and 10 µM of rosiglitazone to the basal media.

4.4. Remove MSC differentiation media and wash the cells using DPBS.

4.5. Add complete adipocyte differentiation media—2 mL for a well of a 6-well plate and 1 mL for a well of a 12-well plate—and incubate the cells at 37 °C. Change complete differentiation media every other day for 14 days.

5. Evaluation of the differentiation efficiency of adipocytes

5.1. On day 14 of differentiation, check the efficiency of differentiation by staining cells for adipocyte maturation markers, FABP4, and adiponectin.

5.2. Remove the media and wash the cells with DPBS.

5.3. Fix the cells using 4% paraformaldehyde (PFA) — 200 µL to a well of a 24-well plate —and incubate at room temperature for 15 min.

5.4. Discard the PFA and wash using tris-buffered saline with 0.5% tween (TBST) and place it on a shaker at room temperature for 15 min. Repeat the process twice.

5.5. Permeabilize the fixed cells with phosphate-buffered saline with 0.5% Triton X-100 (PBST) and place it on a shaker at room temperature for 15–20 min.

5.6. Discard the PBST and add the blocking buffer (5%–6% bovine serum albumin (BSA) in PBST)—500 µL for a well of a 6-well plate and 250 µL for a well of a 12-well plate—and incubate at room temperature on the shaker for 40–60 min.

5.7. Dilute the primary antibodies against FABP4, adiponectin in 2%–3% BSA, at a concentration of 1:500 (see **Table of Materials**). Add these antibodies together only if raised in different animals and place the plate on the shaker at 4 °C, overnight.

5.8. Remove the primary antibodies and wash the cells three times with TBST (15 min each) and place it on a shaker at room temperature.

5.9. Prepare Alexa Fluor secondary antibodies in PBST (1:500). Incubate the cells in the secondary antibody combinations (as per the species in which the primary antibody is raised) for 60 min at room temperature and cover the plate with aluminum foil to protect it from light.

5.10. Discard the secondary antibodies, wash with TBST three times, and place the plate on the shaker.

5.11. To stain the nuclei, add 1 µg/mL of Hoechst 33342—200 µL for a well of a 24-well plate—diluted in PBS and incubate for 5 min at room temperature.

5.12. Discard the Hoechst solution and add PBS—500 µL for a well of a 24-well plate—to the cells. Keep the plates covered from light until visualized using an inverted fluorescence microscope.

6. Sorting of adipocytes using Nile red

6.1. Prepare Nile red working solution by adding 1 mg/mL Nile red stock solution in DMSO and store at -20 °C. Right before use, thaw the Nile red stock and reconstitute in DPBS to attain 300 nM working solution concentration.

6.2. On or after day 14 of adipocyte differentiation, discard the media from the cells and wash using DPBS.

6.3. Add Nile red working solution —1 mL in a well of a 6-well plate— and incubate at 37 °C for 15 min.

6.4. Remove the Nile red solution and add trypsin-EDTA —500 µL in a well of a 6-well plate— and incubate at 37 °C for 4 min.

6.5. Collect the cells using DMEM containing 5% FBS in a 15 mL conical tube. Centrifuge at 750 x *g* for 4 min.

6.6. Remove the supernatant and resuspend in DPBS—1 mL for 1 x 10^6 cells. Centrifuge at 750 x *g* for 4 min.

6.7. Remove the supernatant and resuspend in DPBS—1 mL for 1 x 10^6 cells. Use a FACS sorter to isolate the Nile red-positive cells using the FL1 channel.

6.8. Re-culture the sorted cells in adipocyte differentiation media or collect the sorted cells for RNA and protein isolation.

6.9. Extract RNA from the sorted cells and perform relative quantitative analysis of adipocyte differentiation markers, including *FABP4, PPARG*, and *C/EBPA*. The Nile red-positive cells show a significant upregulation in the gene expression of at least two folds compared to un-sorted cells.

REPRESENTATIVE RESULTS:

Schematic and morphology of cells during mesenchymal differentiation: Differentiation of iPSCs into MSCs involves various stages of development spanning across EB formation, MSC differentiation, and MSC expansion (**Figure 1**). During these stages of development, cells acquire various morphology owing to the different stimulatory chemicals they are subjected to. Upon

initiating differentiation, cells are plated in suspension and are expected to be round, with defined cell borders, while being small to medium size in diameter (**Figure 2**). Choice of culturing cells in suspension during the initial phase of differentiation allows it to closely resemble the process of natural embryonic development, making this phase highly crucial for successful differentiation. The phase of EB formation and RA treatment is followed by plating EBs on basement membrane matrix-coated plates. The viability of EBs upon plating can be accessed by observing their rapid proliferation behavior giving rise to more MSCs (**Figure 2**). This rapid proliferation behavior exhibited by MSCs is retained even after passaging them onto fresh matrix coated plates along with retaining peculiar, elongated morphology (**Figure 2**).

Quantitative assessment of MSC surface markers: Differentiation efficiency of MSCs is accessed by quantification of surface markers specific for MSC differentiation. Good differentiation producing reliable MSCs should show greater than 90% efficiency of mesenchymal surface markers CD73, CD44, and CD90 (**Figure 3A**). In addition to that, cells are also assessed for the absence of surface markers depicting hematopoietic phenotype, CD14, CD34, and CD19, and is therefore expected to show less than 1% expression efficiency for them (**Figure 3B**).

Differentiation of MSCs into adipocytes: Differentiation of MSCs into adipocytes can be accessed by staining for FABP4 and adiponectin. FABP4 is a cytoplasmic protein, and it is regarded as a marker for terminally differentiated adipocytes. Its high expression among adipocytes, with a cytoplasmic distribution, is a key sign of their developmental maturity (**Figure 4A**). In addition to FABP4, adiponectin is regarded as one of the important markers for adipocyte maturity. Its high expression indicates adipocytes are functional enough for undergoing lipid storage and adipogenesis in response to glucose signaling. Being a secretory protein, adiponectin exhibits globular morphology with every protein globule easily distinguishable within the cytoplasm (**Figure 4B**).

Staining and sorting of mature adipocytes using Nile red: Upon differentiation, mature adipocytes can be distinguished from their immature counterparts by staining for Nile red. Nile red binds to lipid-bearing adipocytes, a characteristic exclusive to mature adipocytes (**Figure 5A**). This along with the fluorescent bearing characteristic of Nile red makes it an effective tool for sorting mature adipocytes using fluorescent activated flow cytometry (**Figure 5B**). Effective sorting should result in the enhancement of maturation markers—*PPARG*, *C/EBPA*, and *FABP4*—by at least two folds, determined by quantitative real-time PCR (qRT-PCR) (**Figure 5C**).

FIGURE AND TABLE LEGENDS:

Figure 1: Schematic diagram showing the differentiation of iPSCs into MSCs and adipocytes. iPSCs are differentiated into MSCs using the embryoid body (EB) technique. The EBs are subjected to a short exposure of 10 µM of all-trans retinoic acid (RA). The generated MSCs are differentiated into 40%–77% adipocytes based on the iPSC line. The Nile red positive cells are sorted using FACS to obtain a purified population of mature adipocytes that can be used for studying adipocyte-associated disorders (disease modeling), identifying novel drugs, and eventually for personalized therapy.

Figure 2: **Differentiation of iPSCs into MSCs.** Representative morphological images showing different stages of MSC differentiation at days 2 (D2), 11 (D11), 15 (D15), and 24 (D24). Embryoid bodies (EBs) generated in the presence of 10 µM of RA for 24 h were plated at day 8 of differentiation, followed by dissociation and passaging after 12–17 days of differentiation. The MSCs were passaged several times. Abbreviations: P2 = passage 2.

Figure 3: Expression of MSC markers and hematopoietic markers in iPSC-derived MSCs. Representative flow cytometry histograms showing the expression of the MSC markers, CD73, CD44, and CD90, (**A**) and the hematopoietic markers, CD34, CD19, and CD14 (**B**) in the MSCs generated from iPSC-derived EBs treated with 10 µM of RA. The X-axis in the graph represents the fluorescent intensity.

Figure 4: Differentiation of iPSC-derived MSCs into adipocytes. Immunostaining images showing the expression of FABP4 (**A**) and adiponectin (ADIPO) (**B**) in mature adipocytes derived from iPSCs. The nuclei were stained with Hoechst.

Figure 5: Sorting of iPSC-derived adipocytes using Nile red. (**A**) Images showing bright field (BF) and Nile red-stained mature adipocytes. (**B**) Quantification of Nile red (PE-positive cells) in mature adipocytes using FACS. (**C**) Real-time PCR analysis showing the expression of *C/EBPA*, *FABP4*, and *PPARG* in sorted versus unsorted mature adipocytes. Data are represented as mean ± SD; *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

DISCUSSION:

This protocol holds paramount importance due to its ability to provide MSCs in high yield and efficiency. This mass-scale production of MSCs was made possible by transient incubation of iPSCs-derived EBs with 10 µM of RA[14,15]. Transient treatment with 10 µM of RA enhanced the MSC yield by 11.2 to 1542 folds[14,15], with this protocol being applicable on both iPSCs and hPSCs. At this dose and duration of treatment, RA improves the proliferative and survival capacities of EB-forming cells by direct or indirect regulation of the expression of several genes involved in cell proliferation, apoptosis, and cell-cell and ECM-cell adhesions, which are critical for the survival and proliferation of iPSCs[14,16]. The genes include, but are not limited to, transcription factors (such as EGFR4, SOX4), growth factors and growth factor receptors (such as IGF2, FGFR4), and adhesion molecules (such as FN1 and CAMs). However, in contrast to low doses (0.1–10 µM), at high doses (≥20 µM), RA negatively regulates proliferation and survival of EB-forming cells resulting in reduced PSC-derived EB number and size and thereby a decreased yield of MSCs[14]. RA is regarded as a proliferation inhibitor in several normal differentiated and cancerous cells[17–19]. In EBs, retinoid signaling is context (time, concentration, species, and cell line)-dependent; differentially affecting the self-renewal, survival, and differentiation of EB-forming cells by regulating distinct genes and signaling pathways[20,21]. Therefore, the usage of RA in an optimal time and concentration of RA—10 µM on day 3 of EB induction followed by dose reduction to 0.1 µM on day 5 for 2 days as described in the present protocol—is crucial to induce EB-forming cell survival and proliferation.

In addition to regulating growth and survival, RA does subject the treated EBs to differentiation

delay as compared to cells non-treated with RA[14]. In fact, RA-treated EBs maintain their compact shape after plating and fail to differentiate into MSC-like cells, in contrast to RA-untreated EBs. This is consistent with previous studies reporting that short-term exposure to RA treatment inhibits cell differentiation through the suppression of WNT signaling[21]. Moreover, these RA-treated differentiation-delayed cells also showed enhanced expression of cadherin and extra-cellular matrix proteins[14], which are known to play an important role in maintaining the pluripotent state of iPSCs[16]. To release the RA-mediated differentiation block, EBs should be dissociated, which results in disrupting cell adhesions and allows long-term MSC differentiation upon plating. Interestingly, RA treatment did hold a differentiation block over cells, but it did not maintain the cells in a pluripotent state. In fact, the EB-forming cells undergoing short-term exposure to 10 µM RA show significantly reduced expression of key pluripotency markers—*OCT4, SOX2,* and *NANOG*[14].

The MSCs generated by short-term RA treatment of EBs have been shown to maintain their typical fibroblast-like morphology with abundant expression of MSC surface markers and their multipotency following cryopreservation, thus making these mass-produced MSCs storable for long-term expansion studies[14]. When subjecting them to adipogenic, chondrogenic, and osteogenic differentiation conditions, these MSCs could readily differentiate into the three mesodermal cell types thus making them an easily attainable source for modeling tissue-related diseases[14]. Thus, the stable and versatile *in vitro* behavior of the MSCs generated by the RA-mediated differentiation protocol provides them with paramount importance in research and application-based settings.

While the chondrogenic and osteogenic differentiation potentials of MSCs obtained from RA-treated EBs seem to be similar to those of the MSCs obtained from untreated EBs, the former was found to display an enhanced potential to differentiate into adipogenic lineage when subjected to adipogenic differentiation conditions[14]. This was evidenced by a 2- to 3-fold increase in intracellular lipid accumulation (Oil Red O staining) and adipocyte marker FABP4-positive cells in the differentiation pool of cells obtained after culturing the MSCs derived from RA-treated EBs with adipogenic differentiation media, as compared to MSCs derived from RA-untreated EBs. This could be the consequence of the regulation, by RA, of several signaling pathways governing adipocyte development such as Hippo, WNT, and ECM-cell interaction pathways, as revealed by RNA sequencing data from RA-treated and untreated EBs[14,22–25]. This enhanced ability of RA-derived MSCs to undergo adipogenic differentiation is valuable, as currently available protocols either lead to poor adipocyte yield or make use of genetic manipulation making the generated adipocytes invaluable for deriving natural-process recapitulated adipocytes. Adipocytes are classified into three types—white, brown, and beige. White adipocytes are classified by the presence of a single lipid droplet and play a role in energy storage. Whereas, brown adipocytes are involved in energy expenditure by substrate oxidation due to the very high abundance of mitochondria characterized by the expression of *UCP1*. Whereas the brown adipocytes that are found localized in white adipose tissue are known as beige—or brown-like—adipocytes. These MSC have the potential to give an abundant yield of white adipocytes given the pre-exposure of EBs to RA. Previous publications have stated selective induction of iPSC into cells expressing low *UCP1* i.e., white adipocytes, rather than exposing cells with high *UCP1* levels to RA[26]. Previous

publications have reported that RA produced from neural crest cells in mouse and zebrafish embryos plays an important role in white adipocyte formation[27,28].

Although the RA-based protocol allowed the generation of MSCs that provide increased yield of adipocytes reaching 48.5%–77.4% (vs. 22.5%–57.6% without RA treatment), not attaining >90% is still problematic when modeling multi-variant adipocyte-based genetic disorders *in vitro*. In fact, not reaching a pure adipocyte population could make results coming from multi-variant disease models ambivalent, as it would be hard to distinguish if the observed developmental differences are due to the different genetic makeups or due to inconsistent differentiation efficiencies. In order to circumvent this issue, it was important to sort the differentiated cells to obtain a pool of pure mature adipocytes, so that any differences in phenotypes could only be attributed to inherent genetic differences. Several studies have identified surface markers on adipocytes that could potentially be used for sorting. For example, work carried out by Ronald Kahn allowed the identification of the amino acid transporter ASC-1 as a novel surface marker on white adipocytes[29]. In addition, studies extracting mature adipocytes from omental and subcutaneous regions have reported mature adipocytes to express CD34, CD36, and CD59 on their surfaces[30], where CD36 has been reported to function as a fatty acid transporter on the surface of mature adipocytes[31]. However, these studies have made use of heterogeneous populations of cells derived from the adipose tissue without specifying the expression of these markers to only mature adipocyte populations. Furthermore, these markers can be also expressed by other cell types and are not specific to adipocytes. For example, ASC-1 is present on both astrocytes and neurons[32], CD34 is a marker of hematopoietic stem cells[33], CD36 is present on platelets, mononuclear phagocytes, hepatocytes, myocytes, and some epithelia[33], and CD59 is expressed on endothelial and lymphoid cells[34,35]. Therefore, as an alternative solution, Nile red, the selective fluorescent stain for intracellular lipids, was used as a possible candidate for sorting adipocytes. Adipocytes store a significant bulk of lipids that can be released and used to produce energy, build membranes, or as signaling molecules that regulate metabolism[36]. Nile red dye has previously been used in flow cytometry and microscopy to stain adipocytes derived from murine and human MSCs[37]. Previous studies have reported usage of Nile red for ESC-derived adipocytes and enhancement of adipocyte markers post sorting[38]. The adipocytes generated from the MSCs obtained by the present RA-based protocol were assessed for their ability to be stained by Nile red, indicating their maturity, and sorted to purify them. These Nile red-sorted cells exhibited a two to three-fold increase in the expression of the adipocyte maturation markers, including *PPARG*, *C/EBPA*, and *FABP4* compared to unsorted cells, thus further increasing the yield of iPSCs-derived adipocytes. Although these markers are expressed before lipid accumulation, their expression tags a cell for terminal differentiation to lipid bearing adipocytes. Checking sorting efficiency by these markers allow us to identify a pool where all cells are express *FABP4*, *CEBPa*, and *PPARg*, indicating a pool, which was pre-destined for mature adipocyte formation. Cells are sorted based upon their staining potential to Nile red. Purification efficiency increased by two to three folds due to the high number of adipocytes in the unsorted fraction. The size of lipid bearing adipocytes vary largely during differentiation, where a pool of cells with identical size distribution are sorted. Unsorted fraction encompasses lipid-bearing adipocytes, but they are not fully mature and governed by dissimilar size proportions.

The heterogeneity of MSCs isolated from the human body has been previously reported[39]. This heterogeneity depends on several factors, such as the MSC origin, donors, and conditions[39]. This may lead to variations in their efficiency in treating different diseases. This study suggests that short RA treatment of hPSCs produced under good manufacturing practice (GMP)-compatible culture conditions would give a homogenous population of MSCs. This indicates that the current protocol is a promising approach for generating a large number of clinical-grade MSCs that can be used for MSC-based therapy.

The combination of the RA-based MSC differentiation protocol leading to adipocyte differentiation and Nile red-sorting protocol allowed us to obtain iPSCs-derived adipocytes with enhanced expression of functional markers and increased yield and purity. Thus, this combined protocol would allow the generation, in sufficient quantity and purity, of mature adipocytes from genetically distinct individuals and the potential uncovering of novel genetic variants behind adipocyte-related metabolic disorders.

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DISCLOSURES:

The authors declare that they have no competing interests.

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Figure 1

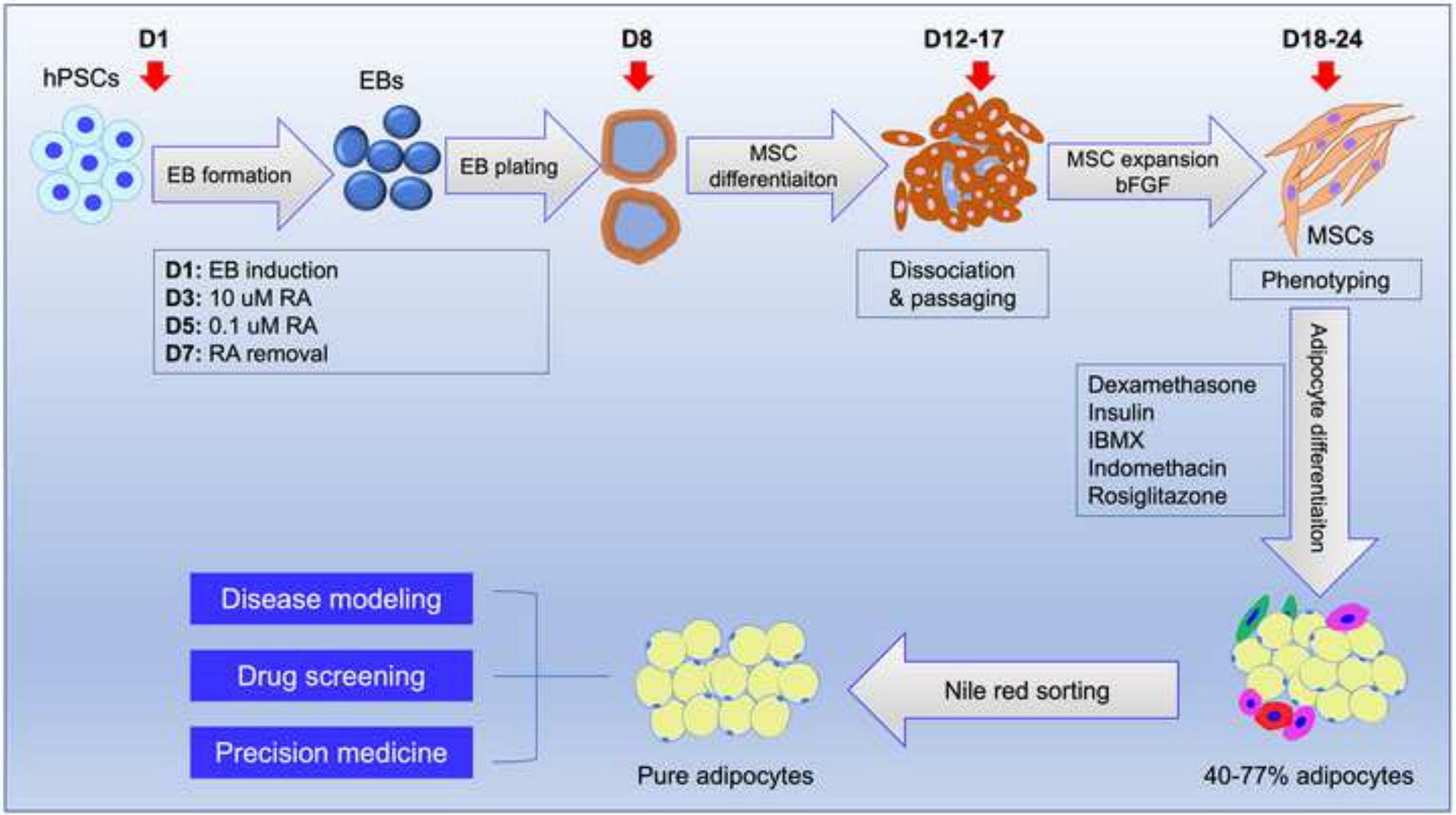


Figure 2

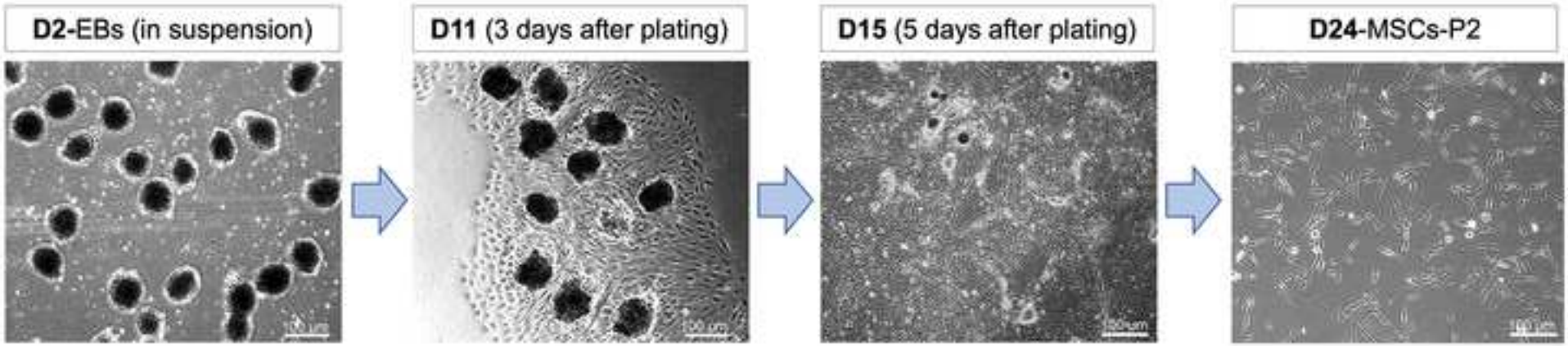


Figure 3

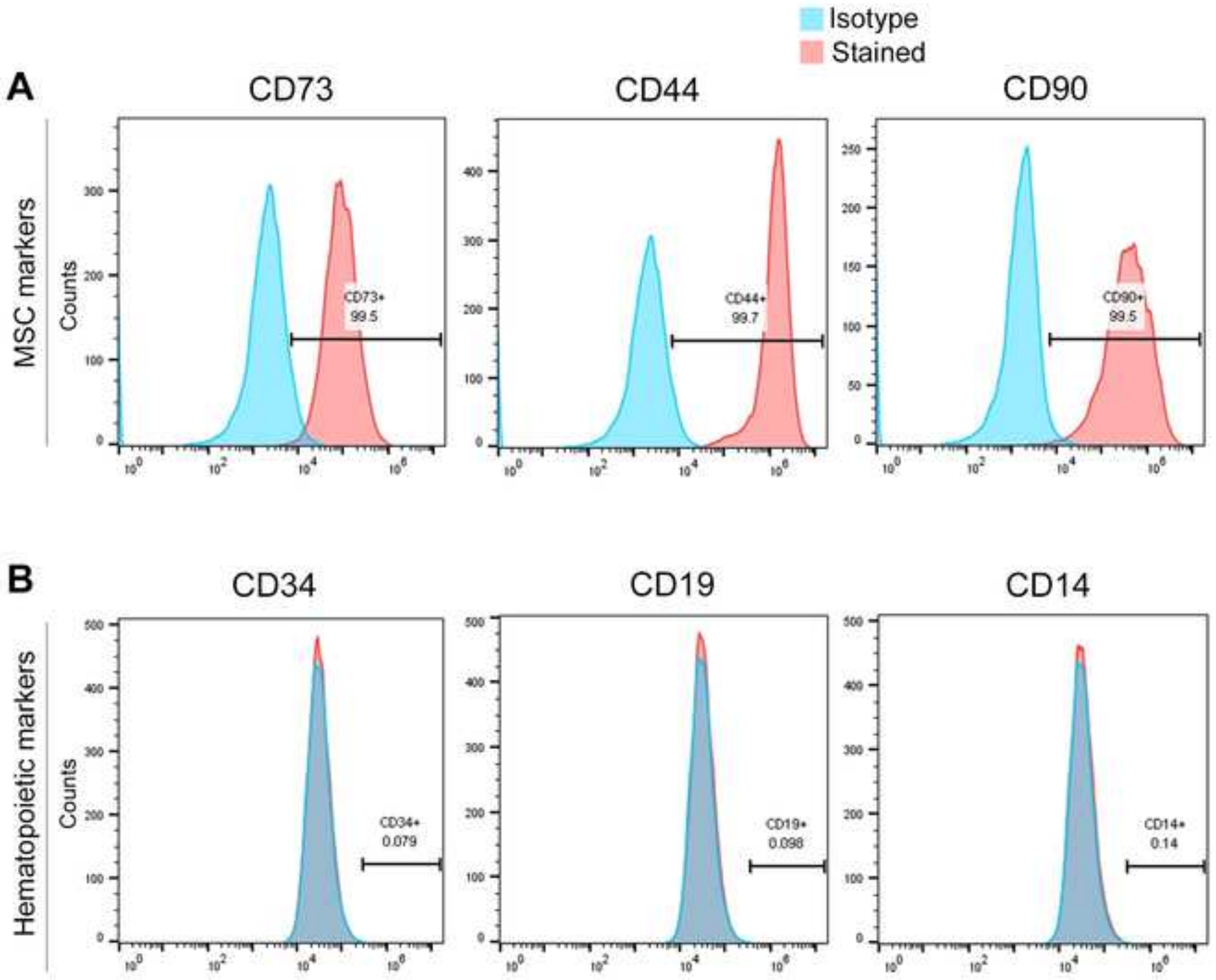


Figure 4

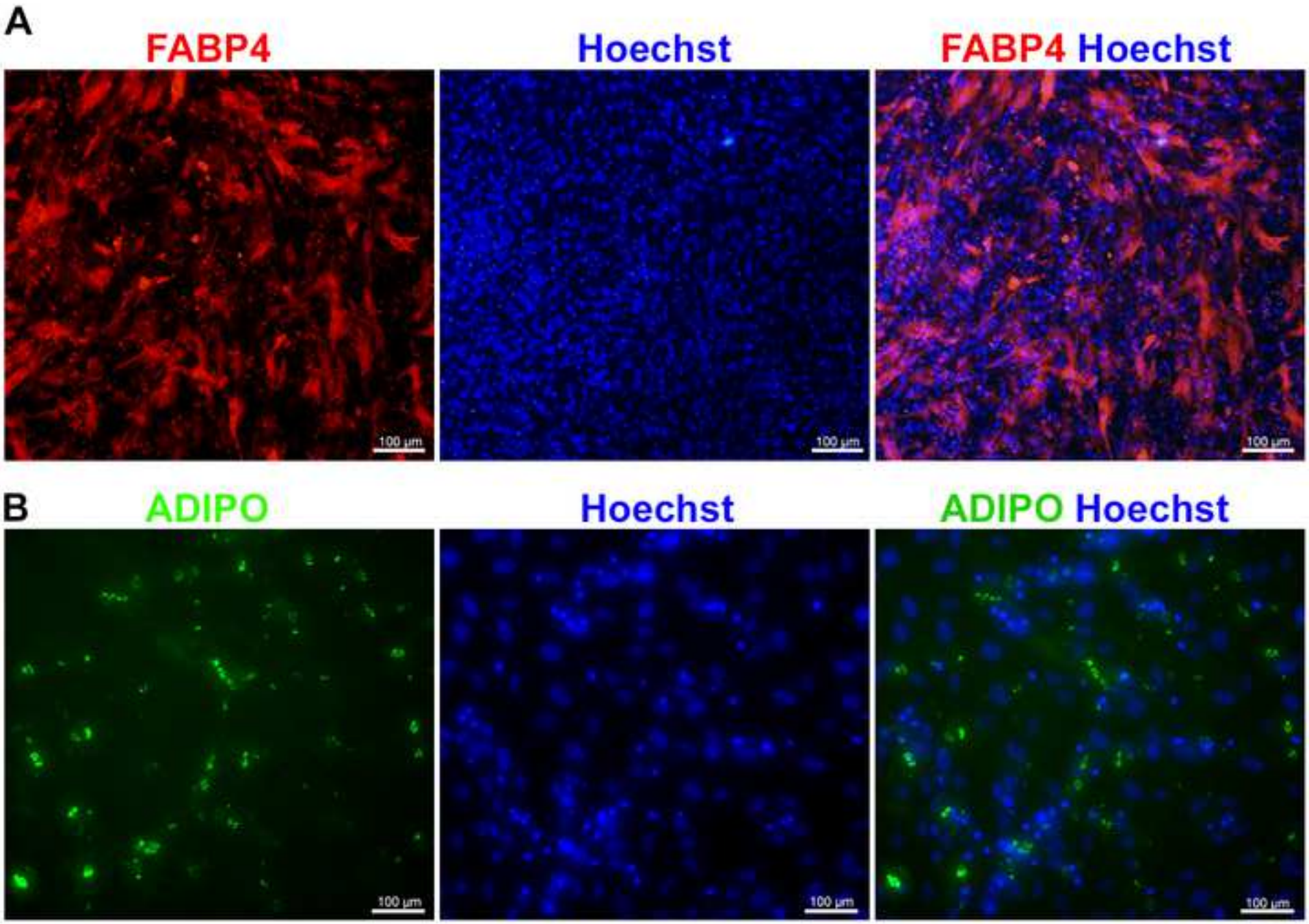
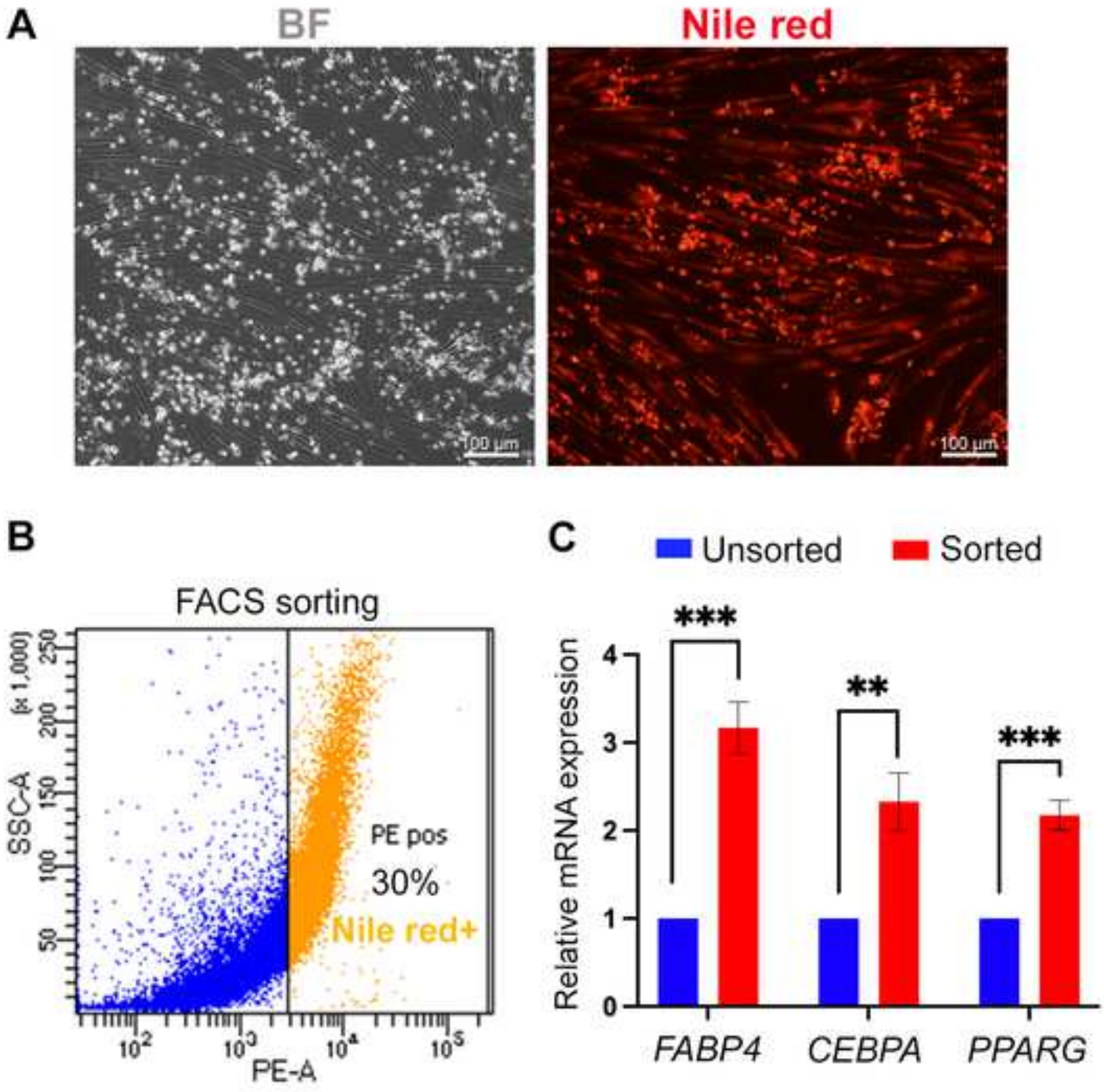


Figure 5



Responses to editor's and reviewer's comments

Editorial comments:
Changes to be made by the Author(s):

Q1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

Yes, we have proofread the document to make sure there are no spelling or grammar issues.

Q2. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials. For example: Geltrex, Matrigel, StemFlex, ReLeSR, Eppendorf, Knockout, ReLeSR, etc.

Yes, we have made sure to remove all trademark from the document.

Q3. For SI units, please use standard abbreviations when the unit is preceded by a numeral throughout the protocol. Abbreviate liters to L to avoid confusion. Examples: 10 mL, 8 µL.

Yes, we have made sure to abbreviate SI units.

Q4. For time units, please use abbreviated forms for durations of less than one day when the unit is preceded by a numeral throughout the protocol. Do not abbreviate day, week, month, and year. Examples: 5 h, 10 min, 100 s, 8 days, 10 weeks.

Yes, I have made sure to abbreviate time durations of less than one day.

Q5. Please note that your protocol will be used to generate the script for the video and must contain everything that you would like shown in the video. Please add more details to your protocol steps. Please ensure you answer the "how" question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. Please add more specific details (e.g., button clicks for software actions, numerical values for settings, etc) to your protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol.
Please specify the volume of medium added.
Line 173, 193: Concentration of isotype, cytokine used.

Concentration of isotype is provided now in line 173 (point: 3.4) (at a concentration of 1:100).

Concentration of cytokines for the formulation of adipocyte differentiation is stated in point 4.3 (page 8).

Line 180,238: Please specify the gating strategy and parameters used for FACS analysis.

Gating strategy is provided in point 3.8.

Line 208: Please specify the dilution of primary antibodies.

Dilution of primary antibody is added now (page 9; point 5.1. 6).

Q6. Please include a one-line space between each protocol step and then highlight up to 3 pages 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. Remember that non-highlighted Protocol steps will remain in the manuscript, and therefore will still be available to the reader.

Yes, one line space has been kept in between each protocol step. Yes, steps required for preparing video have been underlined.

Q7. As we are a methods journal, please also include the following in the Discussion along with citations:
a) Critical steps within the protocol
b) Any limitations of the technique

a) Critical step of this protocol is the transient incubation of cells with high i.e., 10 uM RA and low i.e., 0.1 uM RA for 48 hours, followed by the dissociation of formed EBs. The significance of these steps in thoroughly discussed in paragraph 1 and 2 of the discussion sections. In addition, protocol discusses the usage of Nile red for sorting, whose significance is described in paragraph 5 – pg. 16 of the discussion section.
b) No limitation of the technique is stated.

Q8. Please do not use the &-sign or the word "and" when listing authors in the references. Authors should be listed as last name author 1, initials author 1, last name author 2, initials author 2, etc. Title case and italicize journal titles and book titles. Article titles should start with a capital letter and end with a period and should appear exactly as they were published in the original work, without any abbreviations or truncations.

We have made sure to remove "&" and "and" from author list.

Q9. Figure 2,5: Please include scale bars in all the images of the panel.

The scale bars have been added in Figures 2 and 5.

Q10. Figure 5: Please describe all the symbols used in the graph.

Two symbols were identified in the figure. 1) BF, which is described as bright field in Figure 5 legend. 2) PE, which is described as nile red sorting markers in Figure 5 legend.

Q11. Please ensure that the Table of Materials includes all the supplies (reagents, chemicals, instruments, equipment, software, etc.) used in the study. Please sort the table in alphabetical order. Please remove trademark (™) and registered (®) symbols from the Table of Equipment and Materials.

We have made sure that table of materials encompasses all supplies.

Reviewers' comments

Reviewer #1:

Major Concerns:

Q1. There are two parts of the protocol description that need to be improved. The first one is how to collect iPSCs (point 2.2.2.): is it done by colony picking? The second one is how to perform the FACS staining (point 3.3.). It has to be more precise, including how the gating were performed, please check other Jove protocols that were more detailed (such as Baily et al. "Isolation of Perivascular Multipotent Precursor Cell Populations from Human Cardiac Tissue").

The iPSCs are dissociated using the dissociation reagents (ReLeSR or EDTA) as clearly described in point 2.2.1.

FACS parameters are added as part of point 3.8.

Q2. Lastly, since the described protocol allows the generation of homogenous MSCs, it could be interesting to add few sentences in the discussion describing how this technique could be useful to develop in vitro MSCs based models to study different diseases. In fact, it is well recognized that MSCs, being the in vitro counterpart of perivascular cells are highly heterogenous and show also diverse differentiation abilities (see papers from Vezzani et al. 2016).

Following the reviewer's suggestion, we have added the following sentences "

"The heterogeneity of MSCs isolated from human body has been previously reported. This heterogeneity depends on several factors, such as the MSC origin, donors, and conditions. This

may lead to variations in their efficiency in treating different diseases. This study suggests that short RA treatment of hPSCs produced under Good Manufacturing Practice (GMP)-compatible culture conditions would give homogenous population of MSCs. This indicates that the current protocol is a promising approach for generating large number of clinical-grade MSCs that can be used for MSC-based therapy". (Page 17, last paragraph, marked in red).

Minor Concerns:

Q3. Please decide whether to use iPSCs or hPSCs throughout the text.

Given protocol can be used for iPSC and hPSC both. For the sake of consistency, iPSC is used throughout the text.

Q3. Line 41-43: The word challenge is repeated many times.

The word challenge is replaced by other words.

Q4. Line 44: Please substitute indefinite with a more appropriate word, such as valuable or valid.

Word indefinite is replaced by valuable.

Q5. Lines 56-59 and 62-65: contain the exact repetition of the same concept, using also the same identical words. Please correct.

Lines 62-65 were summarizing everything that was stated above. For the sake of clarity, things have been split into a new paragraph from line 60 onwards.

Q6. Line 60: Typo hPSCs.

Typo has been corrected.

Q7. Line 73: 100 X instead of 1X.

Typo has been corrected.

Q8. Line 77: Please substitute remove (from where?) with take.

From where has been substituted with take.

Line 79: Please add either vial or tube after conical.

Tube has been added after conical.

Line 80: Specify the temperature of the centrifugation.

Temperature has been specified.

Line 116: Induced with the…instead of induced to.

"Induced with the" has been used.

Line 288: Figure 3C do not exist, it should be 5C.

Typo is corrected.

Reviewer #2:

Major Concerns:

Q1. According to the Fig.5C, expression of "mature" adipocytes was only 2-3 times higher in the purified fraction than in the unsorted one. This suggesting either a weak efficiency of the sorting protocol, or a high efficiency of adipocyte differentiation with a high number of adipocytes in the unsorted fraction. If the latter the case asking about the requirement of cell sorting.

Cells are sorted based upon their staining potential to Nile red. Stated efficiency is due to the high number of adipocytes in the unsorted fraction. The size of lipid bearing adipocytes vary largely during differentiation, where a pool of cells with identical size distribution are sorted. Unsorted fraction encompasses lipid bearing adipocytes, but they are not full mature governed by the dissimilar size proportions.

Q2. The choice of the markers FABP4, CEBPa and PPARg could be not appropriate to evaluate the efficiency of adipocyte sorting, since these markers are expressed before lipid accumulation. Therefore, the use of markers expressed in non-differentiated cells and not in adipocytes, such as Hoxc8, CD137 as described in hiPSC-derived adipocyte progenitors by Hafner et al. (Scientific Reports 2016) should be more appropriate.

Although these markers are expressed before lipid accumulation, their expression tags a cell for terminal differentiation to lipid bearing adipocytes. Checking sorting efficiency by these markers allow us to identify a pool where all cells are express FABP4, CEBPa and PPARg, indicating a pool which was pre-destined for mature adipocyte formation.

Minor Concerns:

Q3. Line 107: Authors should describe in more details how to collect clumps formed with 5-10 cells/clump

Clumps are formed by being gentle while dissociation, along with plating the resuspended cells onto a low adherent plate. Point 2.2.2 and point 2.2.5 are rephrased to highlight the significance of these steps.

Q4. The authors indicate that 10 millions of hiPSCs are required for their differentiation into MSCs. In order to evaluate the efficiency of hiPSC differentiation, the number of MSCs obtained afterr 2-3 passages should also be indicated.

Number of MSC at passage 2-3 differs from sample to sample, with it ranging from 3-6 million. Loss of cells is experienced during the stage of EB formation, as some EBs do not remain integrated during differentiation. But this retention rate is better than previously reported, as previous publication by our lab has reported transient treatment with 10uM retinoic acid improves survival potential of EBs (Karam et al., Cell 2020)

Number of expected MSC at passage 2-3 is now added in point 2.6.6.

Reviewer #3:

Major Concerns:

Q1. There doesn't appear to be any experiments that show a downstream application of the protocol. What evidence is there that these purified mature adipocytes respond to a stimulus (insulin for glucose uptake for example, or activation by catecholamines) or can be used for disease modeling or drug screening? What type of adipocytes are these? White? beige? brown?

They are white adipocytes as the differentiation protocol has been directed towards white adipocyte formation. Brown adipocytes are characterized by their thermogenic potential by the expression of UCP1. Conversion of white to brown adipocytes is only possible upon prolong exposure to PPARγ, which was not carried out in this study.

Q2. Has this method been described by another group? It uses an "all-trans retinoic acid" method. Where did this come from? Is this a new method or just an addition on an old method. It is not clear from the introduction and there is no reference as to where they got this previous method or treatment regime.

This differentiation protocol encompassing “all-trans retinoic acid” treatment for generating MSC has been published by our group (Karam et al, Cell 2020). This paper has shown extensive characterization of MSC produced by transient high retinoic acid treatment, by enhancing the survival capacity of EB-forming cells and increasing the expression efficiency of mesenchymal surface markers. Reference for this paper is provided in the discussion section.

Q3. Only old, outdated methods of generating adipocytes from iPSCs are referenced. It's as if the last 10 or so years of research in this field has been ignored. For instance, they say that the efficiency of previous methods is 30-60%. In a paper by Su et al (Cell Reports 25, 3215-3228, December 11, 2018), they obtain nearly 90% PLIN+/PPARG2+ mature adipocytes from a pure population of MSCs. This paper wasn't cited, as well as others.

Paper by Sue et al (Cell Reports 25, 3215-3228, December 11, 2018) describes generation of beige adipocytes from MSC, whereas we are discussing the generation of white adipocytes. Recent years have seen a relative surge in number of publications reporting

differentiation protocol for beige adipocyte formation versus white adipocytes, as brown adipocytes are used for transplantation due to their thermogenic potential of burning fat for improving metabolism.

Q4. The gating in figure 3 for CD73, CD44 and CD90 was very generous. Why is the gate drawn over the blue?

Gate over the blue highlights a very small portion of overlap. Removing it would have no significant effect on the stated efficiency, where it might probably drop from 99% to 96%.

Q5. Article indicates "Choice of culturing cells in suspension during the initial phase of differentiation allows it to closely resemble the process of natural embryonic development, regarding this phase to be highly crucial for successful differentiation." - there is no evidence either shown or referenced that this method recapitulates the process of natural embryonic development other than MSC markers. What type of mesoderm was generated? What is their actual developmental progression?

The embryoid body technique is used to generate several lineages; however, the difference between different protocols is the cytokines used for each lineage. In this study, the cytokines and number of days for each stage are clearly described and published in our previous article with extensive characterization, including FACS, proliferation assays, transcriptome profiling (RNA-sequencing), and real-time PCR (Karam et al, Cell 2020).

Q6. It is not clear if the nile red inside the lipid droplets will have an effect on the phenotype of these cells as there were no downstream functional assays to suggest, that after sorting, that these adipocytes respond as true adipocytes.

Nile red is a hydrophobic compound taken by the cells at standard culture conditions of 37°C. It has been extensively used in staining lipid droplets in live cells in humans, bacteria and eukaryotes, with these studies presenting downstream work of these cells post staining (1, 2). Therefore, it can be safely assumed that Nile red would have no detrimental effects on downstream functional assays performed by adipocytes.

References:
1. Spahn C, Grimm JB, Lavis LD, Lampe M, Heilemann M. Whole-Cell, 3D, and Multicolor STED Imaging with Exchangeable Fluorophores. Nano Lett. 2019;19(1):500-5.
2. Fam TK, Klymchenko AS, Collot M. Recent Advances in Fluorescent Probes for Lipid Droplets. Materials (Basel). 2018;11(9).

Minor Concerns:

1.8.5 Resuspend cells in culture media supplemented with 10 µM ROCK inhibitor and plate them on a fresh matrix coated plates at ratio of 1:3. - poorly written, not correct English

Sentence has been revised.

1.8.4 Collect cells using culture media in a 15 ml 93 conical tube and centrifuge at 800 x g for 4 mins. - A general guideline of how much culture medium to use would be helpful Throughout the article, incorrect use of the term "media". A culture medium is a single type. While there are many different kinds/formulations of media, each one is a medium. I think in most cases you are referring to culture "medium", not media. For example: 2.1 MSC differentiation media: Add 15% Fetal Bovine Serum (FBS) and 1% P/S to Low 100 Glucose DMEM + Pyruvate and store at 4C. Here you are referring to one singular specific type of "medium", not many different types of media.

Media has been replaced with medium.

2.1 Define P/S

P/S has been described in point 1.2

2.2 hPSCs, not hPSC

hPSC has been replaced with hPSCs.

1.8.4 Collect cells using culture media in a 15 ml 93? conical tube and centrifuge at 800 x g for 4 mins.

800 x has been corrected to be 800 rpm.

2.2.3 Centrifuge cells at 800 x g for 4 mins. These speeds of 800g seem very high. Perhaps this is the RPM on your centrifuge? Generally, the fastest cells are spun is around 300g, and iPSCs is around 180g. Is there a reason for 800g? If so explain.

“g” has been changed to “rpm”.

2.3.7 After 48 hours, collect EBs in a 15 ml tube and allow them to settle down for around 15 mins. - remove the word "around" or replace with "approximately". The word "around" is not very scientific. Do the same for other places in the document where this occurs.

Around has been changed to approximately.

2.5.2 Remove the media and resuspend with a fresh 2 ml MSC differentiation media. Perhaps "resuspend with 2 ml of fresh MSC differentiation media" sounds better.

Sentence has been phrased to resuspend with 2 ml of fresh MSC differentiation media.

2.5.5 After 5 days, remove the spent media and replace it by fresh MSC differentiation media containing 2.5 ng/ml bFGF. - replace "by" with "with"

“by” has been replaced with “with”

2.6.1 When plated EBs reach to an 80-90% confluency, passage them. - remove "to an"

“To an” has been removed.

2.6.4 Collect the cells using MSC differentiation media in a 15 ml conical tube and spin at 2000 x g for 4 mins. - This seems way too fast.

MSC are quite small, which require faster centrifugal force.

3.5 Following incubation, centrifuge the plate at 2000 x g for 4 mins at 4 ℃. Discard the supernatant. - Is the supernatant discarded with a multichannel pipette or by shaking the plate down over the sink?

By shaking the plate down the sink.

4.2 Allow MSC to reach above 90% confluency. Continue culturing them for further 48 hours to allow them to undergo a period of growth arrest. - use MSCs (plural) and switch the word "further" for "another"

Further has been changed to another.

4.5 Add adipocyte differentiation media supplemented with cytokines and incubate the cells at 37C. - these are not cytokines. You could just say complete adipocyte differentiation medium.

Instead of cytokines, complete differentiation medium is used.

4.6 Change the differentiation media supplemented with cytokines every other day for 14 days. -again, not cytokines

Instead of cytokines, complete differentiation medium is used.

5.1.6 Dilute the primary antibodies against FABP4, adiponectin in 2-3% BSA (see see Table of Materials). Add each two combined antibodies raised in different animals to the cells and place on the shaker at 4 ℃ overnight. - poorly written

Sentence has been rephrased.