Video Article

Isolation, Expansion, and Adipogenic Induction of CD34+CD31+ Endothelial Cells from Human Omental and Subcutaneous Adipose Tissue

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Abstract

Obesity is accompanied by an extensive remodeling of adipose tissue primarily via adipocyte hypertrophy. Extreme adipocyte growth results in a poor response to insulin, local hypoxia, and inflammation. By stimulating the differentiation of functional white adipocytes from progenitors, radical hypertrophy of the adipocyte population can be prevented and, consequently, the metabolic health of adipose tissue can be improved along with a reduction of inflammation. Also, by stimulating a differentiation of beige/brown adipocytes, the total body energy expenditure can be increased, resulting in weight loss. This approach could prevent the development of obesity co-morbidities such as type 2 diabetes and cardiovascular disease.

This paper describes the isolation, expansion, and differentiation of white and beige adipocytes from a subset of human adipose tissue endothelial cells that co-express the CD31 and CD34 markers. The method is relatively cheap and is not labor-intensive. It requires access to human adipose tissue and the subcutaneous depot is suitable for sampling. For this protocol, fresh adipose tissue samples from morbidly obese subjects [body mass index (BMI) >35] are collected during bariatric surgery procedures. Using a sequential immunoseparation from the stromal vascular fraction, enough cells are produced from as little as 2–3 g of fat. These cells can be expanded in culture over 10–14 days, can be cryopreserved, and retain their adipogenic properties with passaging up to passage 5–6. The cells are treated for 14 days with an adipogenic cocktail using a combination of human insulin and the PPARγ agonist-rosiglitazone.

This methodology can be used for obtaining proof of concept experiments on molecular mechanisms that drive adipogenic responses in adipose endothelial cells, or for screening new drugs that can enhance the adipogenic response directed either towards white or beige/brown adipocyte differentiation. Using small subcutaneous biopsies, this methodology can be used to screen out non-responder subjects for clinical trials aimed to stimulate beige/brown and white adipocytes for the treatment of obesity and co-morbidities.

Video Link

The video component of this article can be found at https://www.jove.com/video/57804/

Introduction

Recent evidence shows that both in mice and in humans, a subset of cells residing in the adipose tissue vasculature can be differentiated into either white or beige/brown adipocytes[1,2,3]. The phenotype of such cells is a subject of controversy, with evidence supporting endothelial cells, smooth muscle/pericyte, or a spectrum of intermediate phenotypes[4,5,6,7]. The scope of developing this methodology was to test the adipogenic potential of CD34+CD31+ endothelial cells isolated from different fat depots from obese humans. Other studies in the literature are focusing on the adipogenic potential of the total stromal vascular fraction or of the known adipocyte progenitors[2,8,9]. Since currently existing technologies can target specifically adipose tissue endothelial cells for drug delivery[10], understanding the potential of such cells to undergo adipogenic induction towards white or beige adipocytes is important for future targeted therapies.

Different groups reported the combination of CD31 and CD34 markers as surrogates to isolate endothelial cells from human adipose tissue[11,12,13]. Typically, the isolation is performed using two sequential steps and a positive selection using magnetic beads. In this report, immunoseparation using CD34+ magnetic beads combined with CD31 plastic beads was utilized. We found this technique superior to the sequential magnetic immunoseparation with respect to the preservation of typical cobblestone endothelial morphology. Also, we were able to generate enough cells required for the expansion and adipogenic induction starting from as little as 1–2 g of fat. A small sample biopsy of subcutaneous fat is enough to produce the required quantity of cells for downstream applications. This aspect is potentially important, particularly if this method will be utilized for screening for a responsiveness to adipogenic induction in human subjects.

Unlike other systems reported in the literature, this method utilizes only two ingredients for the adipogenic induction of the CD34+CD31+ cells: a PPARγ agonist—rosiglitazone—and human insulin. Importantly, the amount of insulin used falls within the normal/high range of circulating post-absorptive insulin in humans[14]. The degree of responsiveness to insulin of the cells *in vitro*, measured by Akt phosphorylation, does not correlate

with their ability to respond to the induction cocktail. Interestingly, using this induction cocktail and experimental conditions, a mix of white and beige/brown cells were obtained as determined by the size and numbers of intracellular lipid droplets and the expression of molecular markers. This straightforward and cost-effective induction protocol along with the quantitative evaluation of the phenotype of the responder cells (white *vs.* beige) allows for a screening of agents that can potentially alter the balance of differentiated beige:white adipocytes.

This method also provides a translational approach for understanding the underlying mechanisms of adipogenesis of vascular endothelial progenitors in human adipose tissue. Using this specific isolation/differentiation technique, investigators can interrogate various pathways responsible for adipogenesis in a subset of vascular endothelial cells from various fat depots in lean and obese humans.

Protocol

The Institutional Review Board Committee at Eastern Virginia Medical School approved the research and collection of human adipose tissue samples used in the study. Informed written consent was collected from the patients.

1. Preparation of Buffers, Media, and Instruments

1. Prepare a Krebs Ringer Bicarbonate-Buffered Solution (KRBBS): 135 mM sodium chloride, 5 mM potassium chloride, 1 mM magnesium sulfate, 0.4 mM potassium phosphate dibasic, 5.5 mM glucose, 1 mM adenosine, 0.01% antibiotic/antimycotic mix (50 µg/mL of penicillin, 50 µg/mL of streptomycin, 30 µg/mL of gentamicin, 15 ng/mL of amphotericin) and 10 mM HEPES (pH = 7.4). This solution is prepared fresh each time for the tissue collection and digestion.
2. Prepare a fresh collagenase solution: 1 mg/mL of collagenase, type 1, in KRBSS; make 3 mL/g of fat. Pre-warm the collagenase solution at 37 °C in a water bath prior to the tissue digestion.
3. Prepare a CD34 Cell Isolation Buffer: 2% fetal bovine serum (FBS) and 1 mM EDTA in sterile phosphate-buffered saline (PBS).
4. Prepare Adipogenesis Media: DMEM/F12, 5% FBS, 50 µg/mL of penicillin/streptomycin, 1.25 µL/mL of human insulin (5 µg/mL or 144 mU/mL), and 0.36 µL/mL of 2.8 mM rosiglitazone (1 µM).
5. Prepare a 0.3% Oil Red O (ORO) stock solution (weight/volume) by dissolving 0.3 g of ORO into 100 mL of isopropanol.

2. Adipose Stromal Vascular Fraction Isolation

NOTE: The study included a cross-sectional cohort of morbidly obese type 2 diabetic (T2D) and non-diabetic subjects, aged 18–65 years, undergoing bariatric surgery at the Sentara Metabolic and Weight Loss Surgery Center (Sentara Medical Group, Norfolk, VA). Exclusion criteria included an autoimmune disease including type 1 diabetes mellitus, conditions requiring chronic immunosuppressive therapy, anti-inflammatory medications, thiazolinendiones, active tobacco use, chronic or acute infections, or a history of malignancy treated within the last 12 months. T2D was defined as a fasting plasma glucose of 126 mg/dL or greater, a glucose of 200 mg/dL or greater after a 2 h glucose tolerance test, or the use of antidiabetic medications.

1. Collect human omental (OM) and subcutaneous (SC) adipose tissue (AT) from human subjects undergoing bariatric surgery.
2. Keep the OM and SC AT in separate vials containing Hank's Buffered Salt Solution with 50 µg/mL of penicillin/streptomycin at room temperature immediately after tissue extraction.
3. Carefully clean and remove fibrotic and cauterized sections of the tissue by using 70% ethanol swabbed scissors and tweezers when the sample arrives at the lab after the tissue extraction.
4. Weigh the adipose tissue and partition it into 5 g (or less) aliquots for the collagenase digestion.
5. Finely mince 5 g of the AT in 5 mL of a collagenase solution in a scintillation vial at room temperature, using two pairs of scissors.
6. Add an additional 10 mL of collagenase solution to the minced tissue for 15 mL total.
7. Digest the samples for 1 h, at 37 °C, in a water bath with continuous shaking.
8. Cut the tip off a 20 mL syringe to make a blunt end.
9. Cut a 9 cm x 9 cm square from a 250 µm mesh and push it halfway into a 50 mL conical tube using the blunt end syringe.
10. Pour the samples into the 20 mL blunt end syringe and filter through the 250 µm nylon mesh into the 50 mL conical tube to separate adipocytes and stromal vascular cells from undigested tissue.
    NOTE: Do not use more than 5 g of AT per 50 mL conical tube, as the mesh will get clogged due to excess fibrotic undigested material.
11. Wash out the scintillation vial with 10 mL of KRBBS and pour it through the filter to collect residual cells.
12. Incubate the filtered samples for 5 min at room temperature.
    Note: This step is important to allow for the adipocytes to float at the top of the tube and some of the stromal vascular cells to settle on the bottom of the tube.
13. Utilize a 20 mL syringe and a 20 G x 6 in pipetting needle to remove the stromal vascular fraction layer and transfer it to a clean 50 mL conical tube.
14. Add 10 mL of KRBBS and allow the samples to sit on the bench for 5 min, at room temperature.
15. Repeat steps 2.12–2.14 twice.
    Note: These steps will ensure that there is no contamination of the stromal vascular cells with the floating adipocytes.
16. Keep the stromal vascular fractions from both depots on ice for further processing, as described in the steps below.
    Note: Do not leave the cells on ice for more than 1 h, as this may have an impact on the cell viability. The adipocytes can be flash-frozen in liquid nitrogen for long-term storage or used fresh for experiments.

3. Isolation of Adipose Tissue Endothelial Cells

1. Spin the stromal vascular fractions (SVF) at 500 x *g* for 5 min, at 4 °C.
2. Remove the samples from the centrifuge and carefully pour off the supernatant; keep the pellet containing the SVF cells.

3. Gently resuspend the cell pellets in 5 mL of PBS.
   NOTE: If more than 5 g of an AT depot was processed into multiple aliquots (see step 2.4), pool the cell pellets together.
4. Spin the samples at 500 x *g* for 5 min, at 4 °C.
5. Remove the supernatant, resuspend the cells in 1 mL of PBS, and count the cells.
   NOTE: Here, an automatic cell counter was used for cell counting. The cell viability was obtained by mixing 12 µL of cell suspension with 12 µL of an acridine orange/propidium iodide (AO/PI) solution (see **Table of Materials**). The average number of cells per gram of AT for each depot is: (a) OM Depot: $1.69 \times 10^5 \pm 4.51 \times 10^4$; (b) SC Depot: $1.24 \times 10^5 \pm 6.45 \times 10^4$. The viability of the cells from both depots was >90%.
6. Pellet the samples at 500 x *g* for 5 min, at 4 °C.
   NOTE: A CD34 immunomagnetic selection protocol (see **Table of Materials**) was adapted for steps 3.7–3.15.
7. Resuspend the pellet in 100 µL of CD34 isolation buffer.
   NOTE: The total cell counts require the following re-suspension volumes: (a) $<2 \times 10^7$ cells: resuspend the pellet in 100 µL; (b) 2×10^8–5×10^8 cells: resuspend the pellet in 1 mL. In steps 3.9–3.16, the volumes of the reagents used were scaled down for $<2 \times 10^7$ cells.
8. Add 10 µL of CD34 cocktail and incubate the sample for 15 min, at room temperature.
9. Add 5 µL of magnetic beads and incubate the sample for 10 min, at room temperature.
10. Add 2.5 mL of CD34 isolation buffer to each sample and place the samples into the magnet, for 5 min, at room temperature.
11. Invert the magnet for 5 s to pour off the isolation buffer.
12. Remove the samples from the magnet and repeat steps 3.10 and 3.11 4x.
13. Remove the tube from the magnet and add 2 mL of CD34 isolation buffer.
14. Pellet the cells at 500 x *g* for 5 min, at 4 °C.
15. Resuspend the cell pellets in 1 mL of CD34 isolation buffer and count the cells.
    NOTE: Here, the average number of cells per gram of AT is: (a) OM Depot: $4.75 \times 10^4 \pm 3.36 \times 10^4$; (b) SC Depot: $1.06 \times 10^4 \pm 9.53 \times 10^3$. A CD31 plastic immunobead protocol was adapted for steps 3.16–3.34 (see **Table of Materials**).
16. Pellet cells at 500 x *g* for 5 min and resuspend the pellet in 250 µL of Buffer B and 250 µL of wash buffer.
17. Thoroughly resuspend the CD31 beads by vortexing the tube.
18. Add 20 µL of CD31 beads.
    NOTE: Due to total cell counts $>1 \times 10^6$, the volume was scaled down according to the manufacturer's input.
19. Incubate the sample with rocking for 30 min, at room temperature.
20. Attach a strainer to a sterile 50 mL conical tube.
    NOTE: The larger opening of the strainer must be on top.
21. Prior to the cell separation, add 1 mL of wash buffer to equilibrate the strainer. Apply the mixture generated under step 3.16 to the strainer.
22. Wash the strainer with 5 mL of wash buffer in a circular motion for a total volume of 20 mL. Attach the connector to a sterile 50 mL conical tube and close the luer-lock.
23. Attach the strainer to the connector. Add 1 mL of wash buffer along the wall of the strainer. Add 1 mL of activated buffer D along the wall of the strainer. Swirl the sample gently and incubate it for 10 min, at room temperature.
24. Add 1 mL of wash buffer. Separate the cells from the beads by pipetting up and down 10x.
    NOTE: Avoid generating air bubbles.
25. Open the luer-lock and allow the detached cells to flow into the 50 mL conical tube. Wash the strainer 10x with 1 mL of wash buffer each time.
26. Discard the connector and strainer and centrifuge the samples at 300 x *g* for 10 min, at 4 °C. Resuspend the cell pellet in 2 mL of complete EGM-2 Media for culture.
    Note: The average number of cells per gram of AT at this point is: (a) OM Depot: $5.92 \times 10^3 \pm 4.27 \times 10^3$; (b) SC Depot: $4.12 \times 10^3 \pm 2.1 \times 10^3$. The viability of cells from both depots was over 90%.

4. Induction of Adipogenesis in Isolated Endothelial Cells

1. Grow the cells in 6-well tissue culture-treated plates with complete EGM-2 media in a 37 °C, 5% CO_2 incubator. Replace the media every 3–4 days until the cells reach 80–90% confluence.
   NOTE: The population doubling is between 2–3 days.
2. Expand the cells by plating them on 100 mm Petri dishes and split the cells 1:3 once. At this stage, the cells are on passage 2.
3. Count the cells using an automatic cell counter (see **Table of Materials**).
4. Seed approximately 100–200,000 cells into 6-well plates ensuring duplicate wells for each depot and culture them in complete EGM-2 media for 2 days.
5. Aspirate off any growth media and replace it with 2 mL of DMEM/F12 with 5% FBS and 50 µg/mL of penicillin/streptomycin for the control cells and replace it with 2 mL of Adipogenesis media (see step 1.4) for the treatment cells.
6. Incubate the cells at 37 °C in a humidified 5% CO_2 incubator for 13 days, replacing the respective media every 3 to 4 days.
   NOTE: Cells will begin to accumulate lipids after 4 days of treatment.
7. Conduct ORO and Nile red staining according to published methods[15-17].
8. To isolate cells for an RNA extraction, remove the media from the 6-well induction plates and wash it with 1 mL of sterile PBS.
9. Add 350 µL of a guanidium thiocyanate-phenol-chloroform solution (see **Table of Materials**) to each well and incubate it at room temperature for 5–10 min.
10. Scrape the cells off the plate using a cell scraper and transfer the cells to a 1.5 mL microcentrifuge tube.
    Note: The samples can be stored in the -80 °C freezer for RNA extraction and further processing at a later date.
11. Extract mRNA from the samples using an adapted RNA extraction protocol (see **Table of Materials**).
12. Perform a real-time polymerase chain reaction (RT-PCR) to assess the gene expression using the following probes: adiponectin, UCP-1, and CIDEA (see **Table of Materials**).
    NOTE: Here, RT-PCR procedures were carried out using the following standard protocol: denaturation (95 °C; 15 s), annealing, and extension (60 °C; 1 min) for 40 cycles. The samples that expressed genes with C_T values >35 were considered undetectable.

Representative Results

Our protocol aims to provide an *in vitro* approach to determine the adipogenic potential of CD34+CD31+ vascular cells from different depots of human adipose tissue. A simplified flowchart diagram is shown in **Figure 1A**. The first step using a positive selection of CD34 expressing cells results in > 95% CD34+ cells in the population of the freshly isolated cells (**Figure 1A**). Importantly, this marker is lost after the cells are cultured for a couple of passages. Since CD34 is a common marker for diverse hematopoietic and non-hematopoietic progenitors, the following step required for the separation of endothelial progenitors is the positive selection of CD31+ cells out of the CD34+ cell population (**Figure 1A**). Although CD31 is a marker for endothelial cells, it can also be found on subsets of hematopoietic cells. We analyzed several preparations from both the omental and subcutaneous fat by flow cytometry and consistently found that CD34+CD31+ cells do not express the CD45 marker (**Figure 1B**, top panels). Typically, <1% of the cells showed CD45 positivity, out of the total cell population. This result led us to conclude that we likely obtained a preparation virtually free of hematopoietic progenitors. To determine if the cells express the adipocyte stem cell marker CD24, we analyzed cells from both omental and subcutaneous depots and found that virtually no cells expressed the CD24 marker in the omental depot (**Figure 1B**, bottom panels) and less than 5% of the cells expressed the marker in the subcutaneous depot (not shown). To conclude, using this separation methodology, we obtained CD34+CD31+CD45-CD24- cells from both omental and subcutaneous depots of obese subjects.

Using the plastic beads conjugated with a CD31 antibody, we obtained cells that displayed the cobblestone endothelial morphology during early passages, as opposed to cells separated using the magnetic beads conjugated with a CD31 antibody that displayed a spindle-shaped mesenchymal phenotype immediately after the separation (**Figure 2A**). To further substantiate the endothelial identity of the CD34+CD31+ cells, we performed two functional assays: an uptake of DiI-Ac-LDL and a basement membrane matrix (referred to as matrix hereon) tube formation *in vitro*. CD34+CD31+ cells were incubated with DiI-Ac-LDL and the great majority of cells were positive for the uptake (**Figure 1C**). As a positive control, we used a human adipose tissue microvascular endothelial primary cell line (HAMVEC) that is commercially available (see **Table of Materials** and **Figure 1C**). We also tested whether the CD34+CD31+ cells retain their endothelial function after culturing by determining the ability of such cells to form spontaneous vessel-like structures *in vitro*, in a 3D-matrix. After 3 passages, CD31+CD34+ forms tubular vessel-like structures in a matrix comparable to a HAMVEC primary human endothelial cell line (**Figure 1D**).

After the expansion of the cells for 2–3 passages, we switched the cells from EGM-2 complete media containing pro-angiogenic growth factors into DMEM/F12 media supplemented with 5% FBS, rosiglitazone (1μM) and insulin (144 mU/mL). Maintaining the cells in EGM-2 complete media dramatically reduced their adipogenic differentiation. Also, an addition of dexamethasone and 3-isobuthyl-1-methylxanthine to the media did not change the rate and the extent of lipid accumulation and an addition of indomethacin significantly reduced lipid accumulation (data not shown). Based on these preliminary experiments, we decided to only use rosiglitazone and insulin for the adipogenic induction. After 14 days of culture in adipogenic media, the cells were fixed and stained with Oil Red O or Nile Red and the nuclei were counterstained with DAPI (**Figure 2B**). In the figures, representative images are shown of cells isolated from paired subcutaneous (SC) (**Figure 2B**, top panels) and omental (OM) (**Figure 2B**, bottom panels) adipose tissue of three human subjects with a BMI between 37–45 kg/m^2. Please note that the response to the induction varies widely between the subjects and between depots of the same subject. As seen in the fluorescent image in **Figure 2B**, a mixed population of unilocular (red arrows) and multilocular (white arrows) lipid-containing cells as well as cells that do not accumulate lipids (yellow arrows) are typically present. The proportion between the numbers of these cells is also highly variable with both the subject and the depot of origin. A quantification of the percentage of lipid-containing cells (based on Oil Red O positivity) out of the total cells (based on DAPI-stained nuclei) showed a significant difference between the SC and OM depots of the same individual, with the cells from the SC depot being more responsive to the adipogenic induction (**Figure 2C**). The explanation for the heterogeneity in response between the subjects and the depots of the same subject is not clear. However, we speculate that is likely related to the intrinsic nature of the cell population that carries the fingerprint of the *in vivo* phenotype/environment and not due to the variability in the isolation protocol.

To confirm that the cells underwent the differentiation to mature adipocytes, we measured the gene expression of the pan-adipocyte marker adiponectin and the brown/beige markers UCP-1 and CIDEA in the cells after 13 days of adipogenic induction compared to un-induced control cells. The adiponectin expression was increased up to 10,000-fold in the differentiated cells compared to the controls (**Figure 2D**). The gene expression of CIDEA and UCP1 was only detectable in the cells following the adipogenic stimulation (**Figure 2D**). In particular, the UCP-1 expression was highly variable, reflecting the different proportions of the white:brown/beige cells within the cell population. The expression of the housekeeping gene RPL27 was remarkably consistent between the samples with C_T values ranging between 18–20 cycles and no significant differences were found between the cells that underwent the adipogenic differentiation and the untreated controls. We also detected a UCP-1 protein expression in the cells that displayed the multi-locular phenotype, in a punctated pattern that suggests a mitochondrial localization (**Figure 2E**). The UCP-1 protein was not detectable in cells that did not accumulate lipids or in cells with multiple, very small droplets that are likely not fully differentiated adipocytes (**Figure 2E**).

Our observation that the adipogenic potential of the CD34+CD31+ cells is depot-specific and highly variable amongst different subjects prompted us to seek for potential correlations with BMI, age, and HbA1c. Amongst the 28 samples tested, we did not find any significant correlations between the adipogenic potential and age or BMI; however, we did find a significant negative correlation between HbA1c and the lipid accumulation in the cells from the OM depot (**Figure 3A**). This finding indicates a potential link with the chronic hyperglycemic environment and deserves future investigation. This also suggests that the CD34+CD31+ cells likely carry the *in vivo* signature of their ability to respond to an adipogenic induction. We also show that these cells display variable levels of Akt phosphorylation following an *in vitro* insulin stimulation (**Figure 3B**, top). However, this variability in response does not correlate well with their ability to undergo an adipogenic differentiation as shown by the Oil Red O staining in the pictures of the matching samples (**Figure 3B**, bottom).

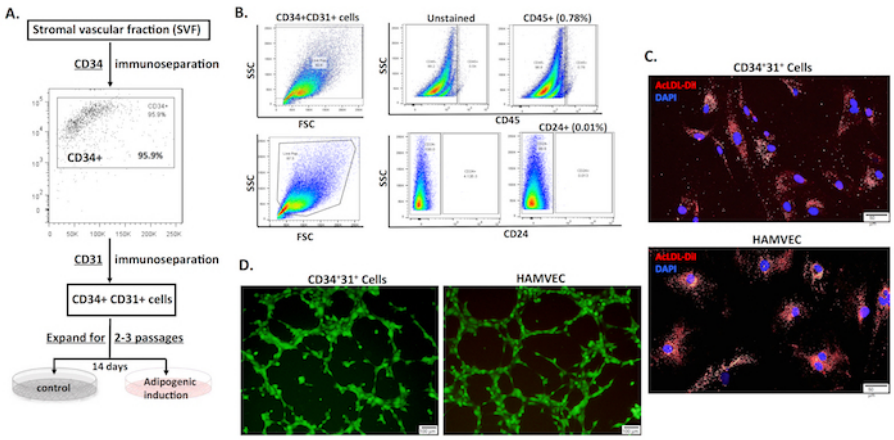

Figure 1: Separation and characterization of CD34+CD31+ cells from human adipose tissue. (**A**) This flowchart diagram shows the major steps of isolation and differentiation. After the positive selection using CD34 magnetic beads, >95% of the freshly isolated cells, prior to the second selection step, are CD34+. (**B**) This representative flow cytometry shows a forward scattered plot gated for live cell population; an unstained sample on the FITC (BluFl2) channel; and CD45 staining of a representative omental sample on top. The bottom shows the same sequence as shown on top, but for the CD24 (Alexafluor 647-labeled) cells from the omental sample. (**C**) These representative micrographs show an uptake of DiI-Ac-LDL (red) by CD34+CD31+ cells following 4 h of *in vitro* incubation (top panel). A similar uptake was found in a human adipose tissue microvascular endothelial cell primary cell line (HAMVEC) (bottom panel). The nuclei are shown blue by DAPI. (**D**) These representative micrographs show an *in vitro* tube formation of CD34+CD31+ cells seeded in a 3D-matrix. CD34+CD31+ cells (passage 3) were seeded in 24-well plates following a staining with FITC-calcein and incubated for 4 h in complete endothelial cell media. The cells were imaged using an inverted fluorescent microscope. HAMVEC, seeded at the same density, was used for comparison. Please click here to view a larger version of this figure.

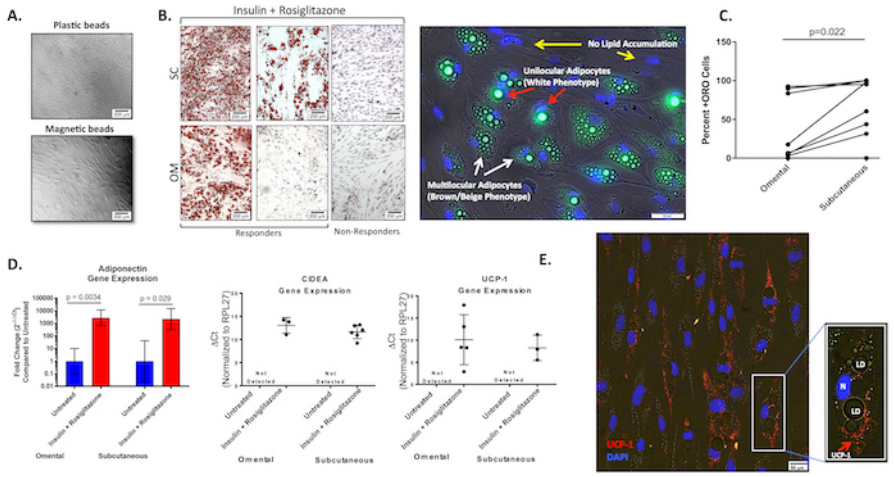

Figure 2: Adipogenic induction and markers of CD34+ CD31+ cells. (**A**) These representative micrographs show the morphologic differences between CD34+CD31+ cells isolated using a positive selection with CD31+ magnetic beads *vs.* CD31+ plastic beads. The cells were imaged at passage 1 after the isolation. The magnification used is 100X. (**B**) These panels are representative micrographs of Oil Red O stained CD34+CD31+ cells from paired subcutaneous (SC) and omental visceral (OM) samples of 3 different subjects. The cells were cultured for 2–3 passages in EGM-2 complete media, then switched on DMEM/F12 media with 5% FBS and treated with insulin (144 mU/mL) and rosiglitazone (1 µM) for 14 days, with media changed every 3 days. The magnification used is 100X. The representative fluorescent image shows adipo red lipid droplets (green) and DAPI nuclear staining (blue). Please note a mix of cells containing unilocular lipids (red arrow), multilocular lipid droplets (white arrow), or cells that show no lipid accumulation (yellow arrow). The magnification used is 200X, Scale bar = 50 µm. (**C**) This panel shows a quantification of adipogenic potential expressed as Oil Red O (ORO) positive cells normalized to total DAPI-stained nuclei. CD34+CD31+ cells from OM and SC depots of the same subject show a significant difference in adipogenic potential by a paired Student's t-test (n = 7). (**D**) The gene expression of mature adipocyte markers (adiponectin, UCP-1, and CIDEA) was measured by RT-PCR in the cells after 14 days of adipogenic induction and in the control cells. The data are expressed as a fold-change for the adiponectin gene expression. The expression of CIDEA and UCP-1 was not detectable in the control samples. The values represent ΔCt normalized to RPL27 as a housekeeping gene. The C_T values for RLP27 were between 18–20 cycles for all the samples included in the analysis (n = 3–5 subjects). The data are expressed as mean ± SD. A paired Student's t-test was used for a statistical analysis of the data. p <0.05 rejects the null hypothesis. (**E**) This panel shows the protein expression of UCP-1 in CD34+CD31+ cells after 13 days of induction with insulin and rosiglitazone. Immunocytochemistry using a human polyclonal UCP-1 antibody showed a selective expression of UCP-1 in multi-locular adipocytes. The detection was achieved using a rhodamine-conjugated secondary antibody (red) and DAPI staining for nuclei (blue). The large magnification in the inset shows the punctated red signal corresponding to UCP-1 (red arrow) as well as the multiple lipid droplets (LD) of different sizes surrounding the cell nucleus (N). Please click here to view a larger version of this figure.

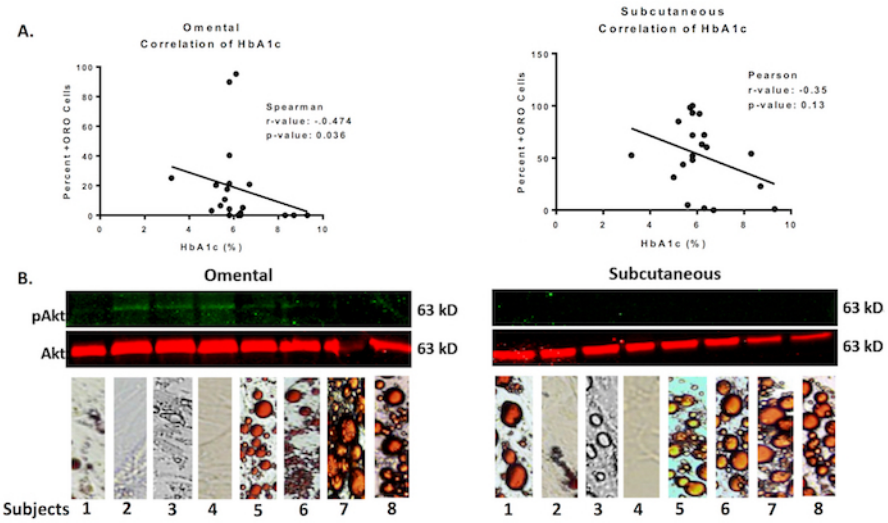

Figure 3: Correlation between adipogenic differentiation, HbA1c and *in vitro* insulin signaling. (**A**) Spearman (non-parametric) and Pearson (parametric) correlation coefficients for the OM and SC cells, respectively, were used to determine the correlation between HbA1c and the lipid accumulation (n = 20). The null hypothesis was rejected for $p < 0.05$. (**B**) At the top, a western blotting shows the phosphorylated Akt (green) and the total Akt (red) expression in CD34+CD31+ cells stimulated *in vitro* with 5 nM insulin for 30 min prior to the adipogenic induction (n = 8). At the bottom, an Oil Red O staining of the same cells following the adipogenic induction for 14 days is shown. Please click here to view a larger version of this figure.

Discussion

The focus of this paper is to provide a methodology for the isolation, expansion and adipogenic induction of CD34+CD31+ endothelial cells from visceral and subcutaneous depots of human adipose tissue.

Methodologies have been reported for the isolation of endothelial cells from various vascular beds of rodents or humans that involve primarily techniques using CD31 antibodies either fluorescently labeled or coupled to magnetic beads[18,19,20,21,22,23]. A major challenge for the universal use of these isolation techniques is the heterogeneity of the endothelial cell population in different vascular beds and the high risk of contamination with fibroblasts and vascular mural cells. However, refinements of the isolation techniques to avoid such contaminations have been reported[24]. Using these techniques, mostly mature endothelial cells are isolated from tissues. Adipose tissue is a large reservoir of stem/progenitor cells, including endothelial progenitors[25,26]. A few papers reported the purification of endothelial cells from human adipose tissue using a combination of CD34+ and CD31+ antibodies[11,27]. Cells that express the two markers are a mixed population of mature endothelial cells and endothelial progenitors[11,28,29]. Several recent seminal papers report that a small subset of endothelial (CD31+) or mural (PDGFRβ+) cells in the adipose vasculature is a rich source of adipocyte progenitors[5,30]. These vascular cells with adipogenic potential can produce both white or brown/beige adipocytes and are associated with active angiogenesis in the adipose vasculature[5]. These findings may provide new therapeutic opportunities to improve the metabolic performance in obesity and/or to induce weight loss by generating white and/or thermogenic adipocytes from vascular progenitors. It is, therefore, of interest to identify a methodology for the easy and economic isolation, expansion, and assessment of the adipogenic potential of vascular endothelial cells from human adipose tissue.

The CD34+CD31+ cells from human subcutaneous and omental visceral adipose tissue depots were previously characterized based on their angiogenic potential, the production of inflammatory mediators, their senescence markers, and other functions[11,31]. However, there is no published evidence for the adipogenic potential of such cells. Previous publications that separated CD34+CD31+ cells using magnetic beads report on data from cells pooled from a large number of subjects (10 or more)[11]. This paper reports for the first time a protocol for the successful isolation and expansion of the CD34+CD31+ cells from single human donors from both the subcutaneous and omental depots. We isolated and expanded cells from as little as 2–3 g of adipose tissue. The CD34+CD31+ cells represented 3–5% of the total SVF cells and displayed >90% viability after the isolation. These cells were highly proliferative and showed a wide range of responses towards the adipogenic differentiation following an *in vitro* stimulation with rosiglitazone and insulin. Previous studies only used adipose stem cells or total stromal vascular fraction from human adipose tissue to test adipogenic responses[4,32,33,34]. One recent study used single cell suspensions from microvasculature of human adipose tissue, but it is unclear whether the differentiated adipocytes were mural or endothelial in nature[3].

Additional immunophenotyping of the CD34+CD31+ cells using flow cytometry showed their lack of CD45 and CD24 markers regardless of their depot of origin. Importantly, we showed that after 3 passages in culture, the CD31+CD34+ cells retain their endothelial functionality demonstrated by the accumulation of acetylated LDL and an *in vitro* tube formation in a 3D-matrix. Also, the differentiated adipocytes represented a mixed population of white and beige/brown cells, based on their morphology and molecular markers, including a UCP-1 protein expression. Previous studies in mice showed that adipose tissue vasculature can be a source of both white and brown adipocytes[5,30] and more recent data showed a similar result with respect to the differentiation of white and functionally thermogenic beige cells from human adipose vasculature[3].

Unlike most publications that used adipogenic cocktails containing multiple ingredients for the adipocyte differentiation from a range of progenitors *in vitro*, we found that rosiglitazone and insulin are necessary and sufficient for the adipogenic induction of CD34+CD31+ cells. While we are aware that rosiglitazone alone can induce a lipid accumulation in non-adipose cells[35], the molecular signature for pan-adipocyte and

brown/beige adipocyte markers, including a UCP-1 protein expression in select cells, confirms the mature adipocyte phenotype of some of the differentiated cells. Our two-ingredient adipogenic cocktail simplifies the procedural protocol and makes it more cost-effective.

An important observation was that the adipogenic response of the CD34+CD31+ cells was highly variable with the donor subject and adipose depot. While depot-specific differences in the adipogenic responses of adipose stem cells or stromal vascular progenitors have been reported previously[32,34], the subject variability of such a response is a novel observation. Out of the 20 human samples analyzed, 3 SC and 7 OM cells did not respond to the adipogenic induction. The most robust responses showed >90% of the cells responsive to the induction for 4 samples from SC and for 2 samples from OM. Interestingly, we found that the responsiveness is negatively correlated with HbA1c and does not correlate with the response to an *in vitro* insulin stimulation in undifferentiated cells. We argue that the difference in response is not due to the poor reproducibility of the technique but rather reflects the individual fingerprint of cells that mimic *in vivo* responses. The preservation of this differential response requires further validation and may represent a relatively easy screening method for the responsiveness to therapies aimed to stimulate adipogenesis *in vivo*. To further understand the source of individual variability in the adipogenic potential, additional immunophenotyping is necessary to identify cell subpopulations within the CD34+CD31+ population that bear a specific adipocyte progenitor function.

Some of the limitations of this protocol include an incomplete characterization of the cell populations; relatively lengthy expansion and differentiation times (2 weeks + 2 weeks); potential limitations to accessing human samples and the need for the immediate processing of the freshly collected tissues; and a current lack of functional data for the differentiated cells. There are several advantages of this methodology that include a reproducible and not labor-intensive protocol; cells that likely retain the fingerprint of their *in vivo* phenotype; the expansion of cells from very small amounts of tissues with relatively low costs; the possibility to cryopreserve the cells and retain their adipogenic signature following re-culture; and the option to generate repositories of these cells so that they can be used for future screening or other purposes.

This protocol and the CD34+CD31+ cells obtained as an end result can be used as a translational platform both for mechanistic studies and for screening purposes.

Disclosures

The authors have nothing to disclose.

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