Video Article

Generation of Human Adipose Stem Cells through Dedifferentiation of Mature Adipocytes in Ceiling Cultures

Julie Lessard[1], Julie Anne Côté[1], Marc Lapointe[1], Mélissa Pelletier[2], Mélanie Nadeau[1], Simon Marceau[1], Laurent Biertho[1], André Tchernof[1,2,3]

[1]IUCPQ Research Center

[2]CHU de Québec Research Center

[3]Laval University

Correspondence to: André Tchernof at andre.tchernof@criucpq.ulaval.ca

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Abstract

Mature adipocytes have been shown to reverse their phenotype into fibroblast-like cells *in vitro* through a technique called ceiling culture. Mature adipocytes can also be isolated from fresh adipose tissue for depot-specific characterization of their function and metabolic properties. Here, we describe a well-established protocol to isolate mature adipocytes from adipose tissues using collagenase digestion, and subsequent steps to perform ceiling cultures. Briefly, adipose tissues are incubated in a Krebs-Ringer-Henseleit buffer containing collagenase to disrupt tissue matrix. Floating mature adipocytes are collected on the top surface of the buffer. Mature cells are plated in a T25-flask completely filled with media and incubated upside down for a week. An alternative 6-well plate culture approach allows the characterization of adipocytes undergoing dedifferentiation. Adipocyte morphology drastically changes over time of culture. Immunofluorescence can be easily performed on slides cultivated in 6-well plates as demonstrated by FABP4 immunofluorescence staining. FABP4 protein is present in mature adipocytes but down-regulated through dedifferentiation of fat cells. Mature adipocyte dedifferentiation may represent a new avenue for cell therapy and tissue engineering.

Video Link

The video component of this article can be found at https://www.jove.com/video/52485/

Introduction

In vitro dedifferentiation of mature adipocytes is achieved through a technique called ceiling culture[1]. Because of their natural tendency to float in aqueous solutions, isolated mature adipocytes adhere to the surface of an inverted flask fully filled with culture medium. Over a few days, cells modify their spherical morphology and become fibroblast-like cells. The resulting cells, called dedifferentiated fat (DFAT) cells, are multipotent[2]. Research articles on adipocyte dedifferentiation, especially on human cells, are limited. However, they have already provided interesting information regarding multipotency, cell phenotype and replicative capacity of DFAT cells[2]. Mature adipocytes originating from various fat compartments have been successfully dedifferentiated including those originating from human visceral and subcutaneous adipose tissues[2-4]. In addition to these depots, Kishimoto and collaborators sampled adipose tissue from the buccal fat pads and dedifferentiated adipocytes into DFAT cells[5]. Matsumoto and collaborators successfully generated subcutaneous DFAT cells from patients covering a wide range of ages, and the majority of cells had a high proliferative rate and less than 6% of senescence even after 10 passages in culture[2].

DFAT cells have been successfully re-differentiated into several lineages, including adipogenic, osteogenic, chondrogenic and neurogenic lineages[2,3,6]. These cells express several embryonic stem cell markers such as Nanog and the four identified pluripotent factors Oct4, c-myc, Klf4 and Sox2[3]. They also express markers specific to each of the three germ layers[7]. In addition, DFAT cells are similar to Bone Marrow-derived Mesenchymal Stem Cells (BM-derived MSC) based on their epigenetic signature[3]. Exploiting the stem cell capacity of DFAT cells, many groups have investigated their potential to treat or improve various diseases[8,9]. Improvements of pathologic conditions, such as infracted cardiac tissue, spinal cord injury and urethral sphincter dysfunction, have been observed with DFAT cell injections in rat models of disease[10-12].

In addition to the stem cell properties of DFAT cells, they may represent a new cellular model for adipocyte physiology studies. The 3T3-L1 cell line is often used for this purpose as these cells differentiate into adherent, lipid-storing adipocytes under adipogenic stimulation[13]. However, these cells originate from mouse embryo tissue[13]. Also, depot-specificity cannot be investigated with this model and it may not fully reflect human adipocyte physiology[14]. Other laboratories work with isolated adipose cells from murine fat depots, but fat distribution is not dimorphic in mice and anatomical configuration of the rodent's abdominal cavity prevents from extrapolating directly to humans[15]. In order to study adipocytes in the context of the physiopathology of human obesity, consideration of body fat distribution and fat depot-specific differences has become essential[16]. Some limitations of primary preadipocyte cultures, including cell quantities obtained from adipose tissue biopsy samples and their senescence after a few passages in culture, created the need for alternate models. Perrini and collaborators investigated depot-specificity in gene expression of DFAT cells originating from visceral and subcutaneous fat and compared them to adipose-derived stem cells (ASC) from the same fat depot. They demonstrated that differences in gene expression and function where mainly found between depots than between cell

types, suggesting that DFAT cells are physiologically close to ASC from the same depot. DFAT cells may represent an interesting alternative to available models for studies on fat distribution in the pathophysiology of human obesity. Moreover, ceiling culture is a promising method to obtain adult stem cells for tissue engineering purposes.

Here, we describe collagenase digestion, a widely-used technique to isolate mature adipocytes from the subcutaneous and/or visceral fat samples[17], and the subsequent steps to perform ceiling culture and dedifferentiate these cells into multipotent, fibroblast-like cells.

Protocol

Ethics statement: The project has been approved by IUCPQ's Research Ethics Committee prior to patient recruitment. For the purpose of this article/video, we obtained tissues from 2 patients: 1) a 62 year-old male patient with a BMI of 50.7 kg/m^2 and 2) a 35 year-old female patient with a BMI of 57 kg/m^2. Experiments can be done with both fat compartments, but have been limited to one fat compartment for the purpose of this video. Technical aspects of the video were performed with patient 1 and FABP4 immunofluorescence was performed with dedifferentiated cells from patient 2.

1. Sample Processing

1. Ask surgeons to collect adipose tissue from the omental and subcutaneous fat compartments at the time of laparoscopic bariatric surgery.
2. Quickly bring adipose samples to the laboratory at RT and process immediately.
3. Perform digestion in the laboratory, in a non-sterile atmosphere. The cells will eventually be transferred to the culture room and cultivated under sterile conditions. To avoid contamination, prepare KRH buffer with distilled and filtrated water and follow by a filtration (0.22µM filter) prior to digestion. Thoroughly clean tubes with ethanol prior transfer in the cell culture hood for flask and plate preparation.
4. Place adipose tissue on a pre-weighted dish and record weight. Fix a small piece of each tissue sample (less than 1 cm^2) in 10% formalin buffer at RT for at least 24 hr before paraffin embedding. Use this embedded sample for immunohistochemistry experiments (technique not shown).
5. Place another piece in a 50-ml tube and flash-freeze in liquid nitrogen before storing at -80 °C for further studies on whole adipose tissues (*e.g.*, gene expression - technique not shown).

2. Collagenase Digestion

1. Place the remaining adipose tissue piece in a 50 ml tube for digestion.
2. Add 4 ml of KRH-WB supplemented with collagenase (350 U/ml) per gram of sample in the digestion tube.
3. Mince adipose tissue with scissors.
4. Place minced adipose tissue suspension in a shaker, 37 °C, 90 rpm maximum, for a 45-minute incubation (maximum 1 hr).

3. Purification of Adipocytes and Preadipocytes

1. Pour the translucent solution with few chunks of fat through a 400 µM nylon mesh into a plastic beaker.
2. With tweezers, rub the cell preparation on the nylon mesh and wash with 5 ml of KRH-WB.
3. Delicately transfer the filtrated cell suspension into a 50 ml tube with the plastic tubing in it and a 60cc syringe attached at the tubing extremity.
4. Let the suspension with mature adipocytes stand for approximately 10 min, allowing the cells to reach the top of the buffer by floatation.
5. Slowly aspirate the buffer at the bottom of the tube using 60cc syringe suction.
6. Add 20 ml of KRH-WB to wash. Repeat from step 3.4 for 2 additional washes.
7. Collect the buffer to bring the adipocyte suspension to a final volume of 5 or 10 ml, depending on cell quantity. Pursue with steps in section 5.
8. Recover the stromal-vascular fraction from the buffer collected with the 60cc syringe by centrifugation (3,000 rpm, RT, 5 min) for further primary cell culture if desired (technique not shown).

4. Mature Adipocyte Cell Count

1. Load 10 µl of gently shaken adipocyte suspension in a counting chamber (haemocytometer). Perform cell count in quadruplicate.
2. Calculate number of isolated mature cells.

5. Mature Adipocyte Dedifferentiation into T-25 Flask

1. Fill a 25 cm^2 tissue culture flask to ¾ of the volume with DMEM/F12-20% calf serum.
2. According to cell count, pour 500,000 mature cells into the flask.
3. Fill the flask completely using a 50ml tube with medium and remove as many bubbles as possible.
4. Screw the unvented cap on the flask.
5. Clean the flask with ethanol prior to incubation to avoid contamination.
6. Incubate the flask upside down for a week without touching it to avoid movement in the culture that may disrupt cellular adherence.
7. Prior to reversing the flask at 7 days of inverted culture, gently manipulate the flask and remove all medium in the flask by aspiration, avoiding abrupt movements.
8. Add 12 ml of DMEM-F12-20% calf serum and cultivate cells with standard techniques. A filtered, vented cap may be added to the flask.

6. Mature Adipocyte Dedifferentiation into a 6-well Plate

1. Place a coverslip on the bottom of each well of a 6-well plate
2. Add a ½" plastic bushing on top of each coverslip.
3. Fill wells with 8 ml of 20% calf serum-DMEM medium.
4. Put a coverslip on each plastic bushing.
5. Insert pipet tip between the slide and the tube to inject cells under the slide (50,000 cells per well).
6. Incubate plates in a standard cell culture incubator at 37 °C with 5% CO_2 for a week.
7. Reverse coverslip with attached cells into each well containing 2 ml of media supplemented with 20% calf serum and pursue culture.
8. Use coverslip with cells undergoing dedifferentiation for several purposes including immunofluorescence (technique not shown).

Representative Results

Major morphological changes occur to mature adipocytes during dedifferentiation (**Figure 1**). As shown in **Figure 2**, cells undergoing dedifferentiation were stained with an anti-FABP4 antibody for fluorescence analysis. Cells with a round morphology expressed the FABP4 protein whereas the majority of the fibroblast-like cells did not. After dedifferentiation, DFAT cells can be cultivated with standard procedures for several passages. We have been able to reach more than 15 passages for human omental and subcutaneous DFAT cell lines (data not shown).

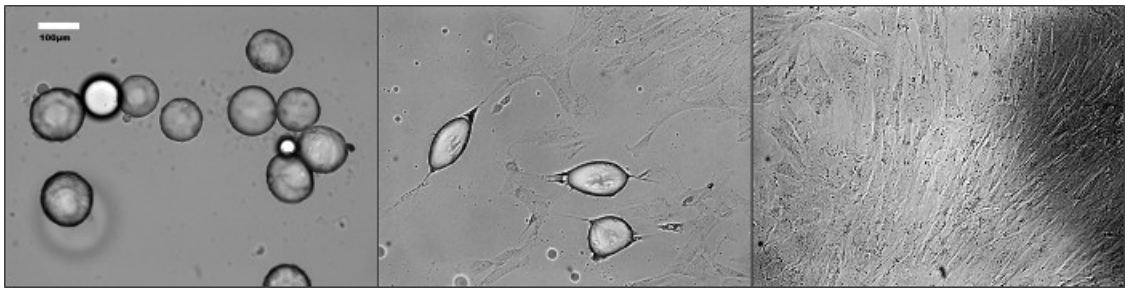

Figure 1. Morphology of dedifferentiating mature adipocytes over time (A) at 4 days (B) at 7 days and (C) at 12 days of culture. Pictures were taken at different time-points during the incubation using a phase-contrast microscope. Please click here to view a larger version of this figure.

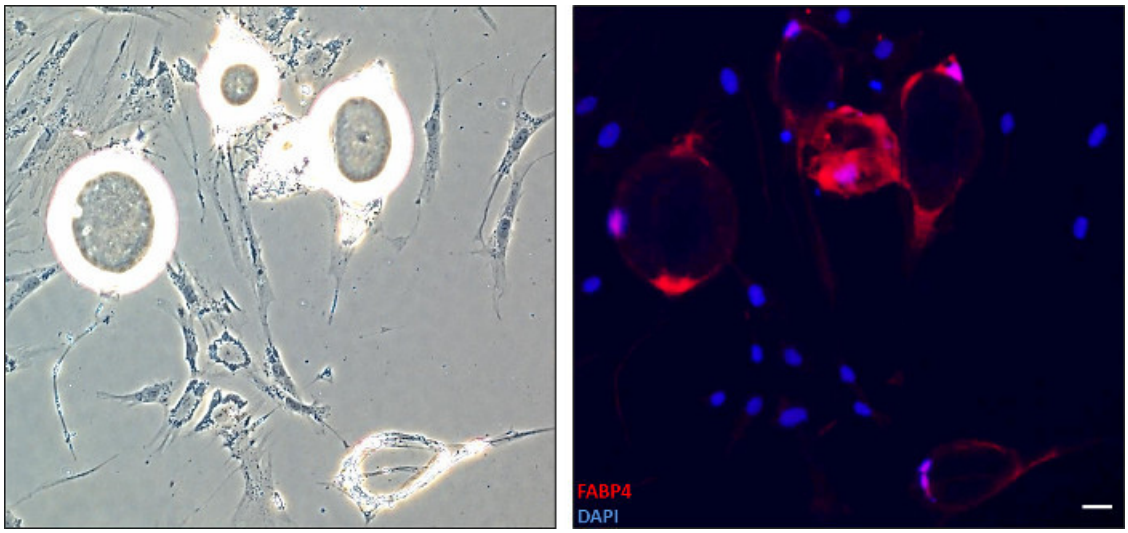

Figure 2. Detection of FABP4 protein in adipocytes undergoing dedifferentiation. Cells were fixed after 13 days of dedifferentiation and stained with anti-FABP4 antibody for immunofluorescence. Nuclei were visualized with DAPI staining. Left: Brightfield image of corresponding immunofluorescence. Right: The merged image is shown (FABP4-red, Nuclei-blue-10X). Adipocytes with round morphology express FABP4, a mature adipocyte marker, whereas elongated cells no longer express it. Scale bar: 1 unit = 0.25mm Please click here to view a larger version of this figure.

Discussion

Dedifferentiation of mature adipocytes with the ceiling culture technique is a new approach to obtain adipose stem cells from a small sample of native adipose tissue. Based on our experience and that of others[2], one gram of tissue is sufficient to plate a 25-cm^2 flask and to obtain a population of DFAT cells for which homogeneity has been demonstrated by Poloni and collaborators[3]. Adipocyte dedifferentiation seems possible with cells from any donor, independently of their age, sex and other characteristics. Among the resulting population of DFAT obtained, there

remains a few round or partly elongated cells that did not fully dedifferentiate. These cells are usually discarded through the culture passage as they float in the trypsin-media mix.

Multipotency of these cells is established and supports their use for cell therapy[2,3]. Their high proliferative capacity has also been reported, which is a valuable aspect of cell culture for stem cell applications[2]. Studies with human DFAT cells indicated that they may be more efficient than ASC from the same donor, based on their replicative and differentiation capacity[18]. A recent case study supports that DFAT cells were more efficient to differentiate into adipocytes and osteoblasts, and had higher telomerase levels than ASC from the same individual, a donor with obesity and diabetes[18]. Thus, the use of ceiling culture may provide more efficient adipose stem cells than the already used ASC. However, additional experiments are needed to clearly assess this point.

Our 6-well plate ceiling culture technique allows for the study of the dedifferentiation process itself. A minimal number of cells can be plated and allows for the study of specific time-points. For example, we collected the microscope slide from a 6-well plate to perform immunofluorescence from adipocytes undergoing dedifferentiation (**Figure 2**). Performing miscroscopy, with or without fluorescence, is highly relevant to assess various aspects of dedifferentiation.

In addition to stem cell applications, DFAT cells may represent an interesting model for physiological studies. Only a few studies examined gene expression and functions of both cell types. In brief, ASC and DFAT from the same fat compartment showed similarities in gene expression and secretion[4]. More comparisons between ASC and DFAT from the same donor are necessary.

In conclusion, we show in this technical report how to obtain DFAT cells from human adipose tissue using the well-established technique of adipose tissue collagenase digestion and the ceiling culture technique. Our original 6-well plate format may help increase knowledge on the dedifferentiation process whereas the more commonly used flask method allows for the generation of larger populations of DFAT cells. The major limitation of this protocol is the access to human adipose tissue which relies on collaboration with a surgery team and ethics management to obtain patient written and informed consent. Mature cells are highly sensitive which requires precautions in the manipulation. We optimized the protocol, especially the number of adipocytes to be plated in the T-25flask and the 6-well plate format, to obtain optimal results and no major additional modifications or troubleshooting may be anticipated.

Disclosures

The authors declare no conflict of interest.

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