Generation of human adipose stem cells through dedifferentiation of mature adipocytes in ceiling cultures

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SHORT ABSTRACT :

Mature adipocytes may represent an abundant source of stem cells through dedifferentiation, which leads to a homogenous population of fibroblast-like cells. Collagenase digestion is used to isolate mature adipocytes from human fat. Ceiling culture is initiated to generate a population of dedifferentiated fat cells with stem cell properties.

LONG ABSTRACT:

Mature adipocytes have been recently 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 up-side 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 plate 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.

INTRODUCTION:

In vitro dedifferentiation of mature adipocyte is achieved through a technique called ceiling culture[1]. Based on the mature adipocyte capacity to float in culture media, cells adhere to the adherent surface of an inverted flask fully filled with culture medium. Over a few days, cells modify their spheric 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. Mature adipocytes originating from various fat compartments have been successfully dedifferentiated including those originating from human visceral and subcutaneous adipose tissue[2-4]. In addition to these depots, Kishimoto and collaborators sampled adipose tissue from the buccal fat pad and dedifferentiated adipocyte 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[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 sencescence 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, and the subsequent steps to perform ceiling culture and dedifferentiate these cells into multipotent, fibroblast-like cells.

PROTOCOL:

1- Reagents and supplies

Prior to adipose tissues sampling, prepare the following reagents and supplies:

1- Liquid nitrogen
2- 10% formalin buffer
3- Sterile tweezer and scissors
4- 60cc syringe and tubing (one per biopsy site)
5- Krebs-Ringer-Henseleit stock buffer (KRH) (25mM HEPES pH7.6, 125mM NaCl, 3.73mM KCl, 5mM $CaCl_2.2H_2O$, 2.5mM $MgCl_2.6H_2O$, 1mM K_2HPO_4) pH is adjusted to 7.4
6- Krebs-Ringer-Henseleit-Working Buffer (KRH-WB) add the following components freshly to KRH buffer: (4% bovine serum albumin, 5mM glucose, 0.1µM adenosine, 560 µM ascorbic acid)
7- KRH-WB supplemented with Type I collagenase (350U/ml)
8- DMEM/F12 medium-20% calf serum (with fungizone and gentamicin)
9- T25 unvented cap tissue culture flask
10- 6-well tissue culture plate
11- Cover slip
12- ½" plastic bushing (Iberville, Memphis, TN)
13- Microscope

2- Sample processing

2.1) The project has been approved by IUCPQ's Research Ethics Committee prior to subject's recruitment. The surgeon collected adipose tissue from the omental and subcutaneous fat compartments at the time of laparoscopic bariatric surgery. 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. Adipose samples were quickly brought to the laboratory at room temperature and processed immediately.

2.2) The digestion is performed in the laboratory in a non-sterile atmosphere after which the cells are transferred to the culture room and cultivated under sterile conditions. KRH buffer must be prepared with distilled and filtrated water and followed by a filtration (0.22µM filter) prior to digestion. Tubes are thoroughly cleaned with ethanol prior transfer in the cell culture hood for flask and plate preparation. With these precautions, contamination is avoided.

2.3) 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 room temperature for at least 24 hours before paraffin embedding. This embedded sample can be used for

immunohistochemistry experiments (technique not shown). 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).

3- Collagenase digestion

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

4- Purification of adipocytes and preadipocytes

4.1) Pour the translucent solution with few chunks of fat through a 400 µM nylon mesh into a plastic beaker.
4.2) With tweezers, rub the cell preparation on the nylon mesh and wash with 5 ml of KRH-WB.
4.3) 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.
4.4) Let stand mature adipocytes approximately 10 minutes, allowing the cells to reach the top of the buffer by floatation.
4.5) Slowly aspirate the buffer at the bottom of the tube using 60cc syringe suction.
4.6) Add 20 ml of KRH-WB to wash. Repeat from step 4.4 for 2 additional washes.
4.7) 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.
4.8) Recover the stromal-vascular fraction from the buffer collected with the 60cc syringe by centrifugation for further primary cell culture if desired (technique not shown).

5- Mature adipocyte cell count

5.1) Use a counting chamber (haemocytometer)
5.2) Load 10 µl of gently shaken adipocyte suspension in each chamber. Perform cell count in quadruplicate.
5.3) Calculate number of isolated mature cells.

6- Mature adipocyte dedifferentiation into T-25 flask

6.1) Fill a 25 cm^2 tissue culture flask to ¾ of the volume with DMEM/F12-20% calf serum.
6.2) According to cell count, pour 500 000 mature cells into the flask.
6.3) Fill the flask completely using a 50ml tube with medium and remove as much bubbles as possible.
6.4) Screw the unvented cap on the flask.
6.4) Clean the flask with ethanol prior to incubation to avoid contamination.
6.5) Incubate the flask up-side down for a week without touching it to avoid movement in the culture that may disrupt cellular adherence.

6.5) 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.

6.6) Add 12 ml of DMEM-F12-20% calf and cultivate cells with standard techniques. A filtered- vented-cap may be added to the flask.

7- Mature adipocyte dedifferentiation into a 6-well plate

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

REPRESENTATIVE RESULTS:

Major morphological changes occur to mature adipocytes during dedifferentiation. As shown on Figure 1, we stained cells undergoing dedifferentiation 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 LEGEND:

Figure 1: 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. The merged image is shown (FABP4-red, Nuclei-blue-10X). Adipocytes with a round morphology express FABP4, a mature adipocyte marker, whereas elongated cells not longer express it.

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]. Multipotency of these cells is established and supports their use for cell therapy[2,3]. Studies with human DFAT cells indicated that they may be more efficient than ASC from the same donors, based on their replicative and differentiation capacity[17]. A recent case study supports showed 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[17].

Our 6-well plate ceiling culture 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 1). 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 a larger populations of DFAT cells.

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DISCLOSURES

The authors declare no conflict of interest.

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