

Primary adipocyte culture: adipocyte purification methods may lead to a new understanding of adipose tissue growth and development

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Abstract

In the present manuscript, the methods required to generate purified cultures of mature adipocytes, as well as stromal vascular cells, from the same isolation are detailed. Also, we describe the *in vitro* conditions for the dedifferentiation of the isolated mature adipocytes. These two types of cells may be used to reevaluate differences between presently available cellular models for lipogenesis/lipolysis and might provide a new cellular physiological system for studies utilizing the proliferative progeny from mature adipocyte dedifferentiation. Alternative possibilities to the dedifferentiation phenomenon are proposed, as this new area of research is novel.

Abbreviations: DMEM – Dulbecco's modified Eagle medium; DMEM/F12 – 1:1 ratio; Dulbecco's modified Eagle medium + Ham's F12; FBS – fetal bovine serum; HBSS – Hank's balanced salt solution; HS – horse serum; PBS – phosphate buffered saline, pH 7.08; PSG – pigskin gelatin; SC – satellite cell

Introduction

The cellularity of adipogenesis has been questioned, since mature adipocytes possess the ability to proliferate and form populations of proliferative-competent progeny cells (Fernyhough et al. 2005a). This previous communication has led to descriptive papers, which discuss the physiological significance of such plasticity displayed by the seemingly differentiated mature adipocyte (Dodson et al. 2005; Fernyhough et al.

2005b). However, exact methods that might allow researchers to isolate absolutely pure populations of mature adipocytes for subsequent research endeavors have not been published. As such, in the present communication, we provide detailed methods, which will allow for the isolation of purified cultures of mature adipocytes from beef-derived fat tissue, and their subsequent progeny cell cultures. These same isolation procedures may be used for adipose tissue from any other animal.

Materials

A. Major equipment

1. Autoclave, gravity air remover type, model P-89501-091.¹
2. Balance, model A-160.²
3. Centrifuge, model TJ-6.³
4. CO₂ water-jacketed incubator, model NU-4500.⁴
5. Dry heat gravity oven, model 1370 GM.⁵
6. Laminar flow, biological safety cabinet, Labguard Class II, Type A/B3, model NU-425-4000.⁴
7. Microscope, Diaphot-TMD phase inverted.⁶
8. Peristaltic pump, model 7015-72.⁷
9. pH electrode, model 47636.⁸
10. pH meter, digital, model 430.⁸
11. Pipet Aid, model 174.⁹
12. Propane torch, Bernz-o-matic model TS2000.¹⁰
13. Water bath stainless steel, model 185.¹¹

B. Media, reagents, and chemicals

1. Bovuminar protease-free BSA, product number 3100-01.¹²
2. Collagenase type I, product number 17100-017.¹²
3. Dulbecco's modified Eagle's medium (DMEM), product number 12800-017.¹²
4. Dulbecco's modified Eagle's medium (DMEM)/Ham's F12 (F12), product number 12500-062.¹²
5. Ethylenediamine tetraacetic acid (EDTA), product number 15576-028.¹²
6. Fetal bovine serum (FBS), product number 26140-079.¹²

7. Hank's balanced salt solution (HBSS), product number H-1387.¹³
8. HCl, product number H-1758.¹³
9. Horse serum (HS), product number 16050-114.¹²
10. Gentamicin, 10 mg/ml gentamicin sulfate, product number 15710-064.¹²
11. Giemsa stain, product number GS-1L.¹⁰
12. D-(+)-Glucose, product number G-7021.¹⁰
13. KCl, product number P-5405.¹⁰
14. KH₂PO₄, product number P-5655.¹⁰
15. NaCl, product number S-5886.¹⁰
16. NaHCO₃, product number S-5761.¹⁰
17. NaOH, pellet, product number 3722-I.¹⁴
18. Na₂PO₄, product number S-5136.¹⁰
19. OptiPrep[®], product number D1556.¹⁰
20. Penicillin-streptomycin (pen/strep), liquid; contains 10,000 units of penicillin G (sodium salt) and 10,000 μg of streptomycin sulfate/ml in 0.85% saline, product number 15140-122.¹²
21. Trypsin, product number T-4549.¹⁰

C. Glassware

1. Media bottles
 - a. 125 ml with cap, product number 219715.¹⁵
 - b. 250 ml with cap, product number 219717.¹⁵
 - c. 500 ml with cap, product number 219719.¹⁵
2. Pipettes
 - a. 2 ml, glass disposable, product number 72120-21100.¹⁶
 - b. 5 ml, glass disposable, product number 53283-774.¹⁷
 - c. 10 ml, glass disposable, product number 53283-776.¹⁷
 - d. Pasteur, non-plugged, glass, 5 3/4" × 1/10", product number 14672-200.¹⁷
3. Volumetric flask, 1000 ml, product number 2813-1000.¹⁶

¹American Sterilizer Company, Erie, PA, USA.

²Denver Instrument Co., Arvada, CO 80004, USA.

³Beckman Instruments Inc., Palo Alto, CA, USA.

⁴Nu Aire Inc., Plymouth, MN, USA.

⁵The Newell Group, Medina, NY, USA.

⁶Nikon, Nippon K. K., Tokyo, Japan.

⁷Millipore Corp., Bedford, MA, USA.

⁸Corning Incorporated Life Sciences, Acton, MA USA.

⁹Drummond Scientific Co., Brownhill, PA, USA.

¹⁰Sigma Chemical Company, St Louis, MO, USA.

¹¹Precision Scientific, Chicago, IL, USA.

¹²Invitrogen/Life Technologies, Carlsbad, CA USA.

¹³Sarstedt, Newton, NC, USA.

¹⁴JT Baker, Paris, KY, USA.

¹⁵Wheaton Scientific Products, Millville, NJ, USA.

¹⁶Kimble/Kontes, Vineland, NJ, USA.

¹⁷VWR Scientific Products Corporation, Chester, PA, USA.

D. Plastic supplies

1. Beakers 1000 ml, polypropylene, product number 1201-1000.¹⁸
2. Centrifuge tubes
 - a. 50 ml, product number 352070.¹⁹
 - b. 15 ml, product number 352096.¹⁹
3. Tissue culture flask, 25 cm², product number 83.1810.001.¹⁰
4. Tissue culture flask, 12.5 cm², product number 353018.¹⁹

Procedures

A. Preparation of reagents and media used in cell culture

1. Preparation of PBS

- a. Dissolve 10.0 g NaCl, 0.25 g KCl, 1.44 g Na₂HPO₄ in 900 ml of glass distilled H₂O.
- b. Adjust to pH to 7.08 with HCl/NaOH.
- c. Bring volume to 1 l in a volumetric flask.
- d. Aliquot into 2–500 ml bottles and autoclave 30 min at 121 °C at 15 psi.
- e. Store at 4 °C.

2. Preparation of trypsin solution

- a. Prepare PBS/EDTA by adding 0.168 g Na₂EDTA·2H₂O to 1 l of PBS.
- b. Sterilize by autoclaving for 30 min at 121 °C at 15 psi.
- c. Add 10 ml sterile trypsin to 90 ml sterile PBS/EDTA.
- d. Store frozen.

3. Preparation of DMEM

- a. Dissolve a 1-l envelope of DMEM and 3.7 g NaHCO₃ in 900 ml of glass distilled H₂O.
- b. Adjust pH to 7.08 with HCl/NaOH.
- c. Bring the volume to 1 l in a volumetric flask.

- d. Filter sterilize in the laminar flow hood by passing the medium through a 0.22 μm pore filter into a sterile 1-l bottle.
- e. Store at 4 °C.

4. Preparation of DMEM/F12

- a. Dissolve a 1-l envelope of DMEM/F12 and 2.438 g NaHCO₃ in 900 ml of glass distilled H₂O.
- b. Adjust pH to 7.08 with HCl/NaOH.
- c. Bring the volume to 1 l in a volumetric flask.
- d. Filter sterilize in the laminar flow hood by passing the medium through a 0.22 μm pore filter into a sterile 1-l bottle.
- e. Store at 4 °C.

5. Preparation of HBSS

- a. Dissolve a 1-l container of HBSS and 0.35 g NaHCO₃ in 900 ml of glass distilled H₂O.
- b. Adjust pH to 7.08 with HCl/NaOH.
- c. Bring the volume to 1 l in a volumetric flask.
- d. Filter sterilize in the laminar flow hood by passing the medium through a 0.22 μm pore filter into a sterile 1-l bottle.
- e. Store at 4 °C.

6. Preparation of HBSS + antibiotics/antimycotics

In the laminar flow hood, supplement the HBSS with antibiotics and antimycotics by adding 1% Pen/Strep, 0.5% ml Gentamicin, and 0.001% (1:1000 dilution) of antimycotic to sterile HBSS. Store at 4 °C.

7. Basal medium + 10% serum (FBS or HS)

In the laminar flow hood, prepare 500 ml of serum-containing medium by adding 50 ml of serum, 5.0 ml of pen/strep, 2.5 ml gentamicin to 442.5 ml of basal medium. Store at 4 °C.

8. Preparation of the enzyme solution

Measure 100 ml of PBS into a glass beaker and place on a stir plate. Add 1.5 g BSA and 90.1 mg glucose. For this procedure, any good quality BSA would likely be acceptable. As we are eventually going to devise a completely defined system to evaluate fractions of isolated cells, we used a form of BSA that was protease and fatty acid free. Stir until all BSA is dissolved then filter through a 0.22 μm pore vacuum filter. Just before use, add

¹⁸Nalge Nunc™, Rochester, NY.

0.25 g collagenase to warmed (37 °C) PBS solution and sterile filter through a 0.22 μm pore vacuum filter. The final concentrations of the solution are 0.25% collagenase, 5 mM glucose, and 1.5% BSA in PBS.

Experimental Procedures

Cell isolation

Adipocytes and cells possessing similar buoyant densities are initially isolated and cultured as a modification of an earlier method (Sugihara et al. 1986; Figure 1a).

1. Transport the adipose tissue slice from the abattoir (since this isolation is for beef-derived fat tissue; if smaller animals are used, then the tissue isolation may be obtained in the tissue culture laboratory) to the cell culture laboratory in sterile 37 °C HBSS supplemented with antibiotics and antimycotics.
2. In a laminar flow hood, place the tissue in a sterile 150 mm dish, bath in HBSS, and cut into approximately 1-cm² pieces using sterile scissors and thumb forceps.
3. Place approximately 5 g of tissue into each of the four 50 ml centrifuge tubes (20 g tissue total). Pipette or pour 25 ml sterile enzyme solution to each tube. Place the tubes on a rocker and incubate 1 h at 37 °C.
4. After enzymatic cell dispersal, filter the tissue in each tube through a sterile 1000 μm plastic mesh in a sterile funnel into a clean sterile 50 ml centrifuge tube.
5. Spin the filtrate in a centrifuge for 10 min at 186 $\times g$. In the laminar flow hood, remove and discard the underlying pellet (containing preadipocytes, fibroblasts, and erythrocytes) and media. If the stromal vascular cells are wanted, remove the underlying pellet and transfer to a tissue culture flask containing serum-containing medium. Re-suspend the remaining fat layer in 20 ml HBSS and centrifuge for 10 min at 186 $\times g$. Repeat this step two additional times.
6. After the last centrifugation step, transfer the fatty layer (containing the mature adipocytes) to 12.5 cm² cell culture flasks (one flask per

tube). Fill the flasks completely with a 1:1 mix of DMEM/F12 + 10% HS.

7. Invert the flasks so that the bottom of the flask is on top. This allows the floating unilocular adipocytes to attach to the upper portion of the flask and any remaining fibroblast-like cells to sink to the bottom. Incubate the flasks at 37 °C in a 5% CO₂ incubator and monitor daily for cell attachment. There will be residual cells that contaminate the floating cell compartment, and these will need to be removed from the cultures in order to insure a homologous population of cells for research purposes. The removal of the contaminating cells is vital for subsequent success in culturing mature fat cells and determining that mature fat cells are capable of dedifferentiating to form proliferative-competent cells.
8. After sufficient attachment of the cells (usually 5–7 d) remove the medium, replace with 5 ml fresh medium, and re-invert the flasks. This allows for the normal observation and subsequent manipulation of the cultures.

Purification of isolated cells

Differential plating

Two methods of differential plating are employed to insure the purity the mature adipocyte cultures. The first method (early differential plating; Figure 1b) exploits the extended time needed for the unilocular adipocytes to attach to the cell culture surface, as compared to any preadipocyte and fibroblast that might have been co-isolated and cultured. The second method of differential plating (late differential plating; Figure 1c) involves cultures that are firmly adhered to the flask (i.e. greater than 4 d in culture).

Early differential plating

1. Remove the medium from each flask and transfer to a clean flask.
2. Add additional serum-containing medium (if needed) to fill the flask.
3. Place each flask once more in ceiling culture. This transfer will allow the more tenuously attached mature adipocytes to detach (and be

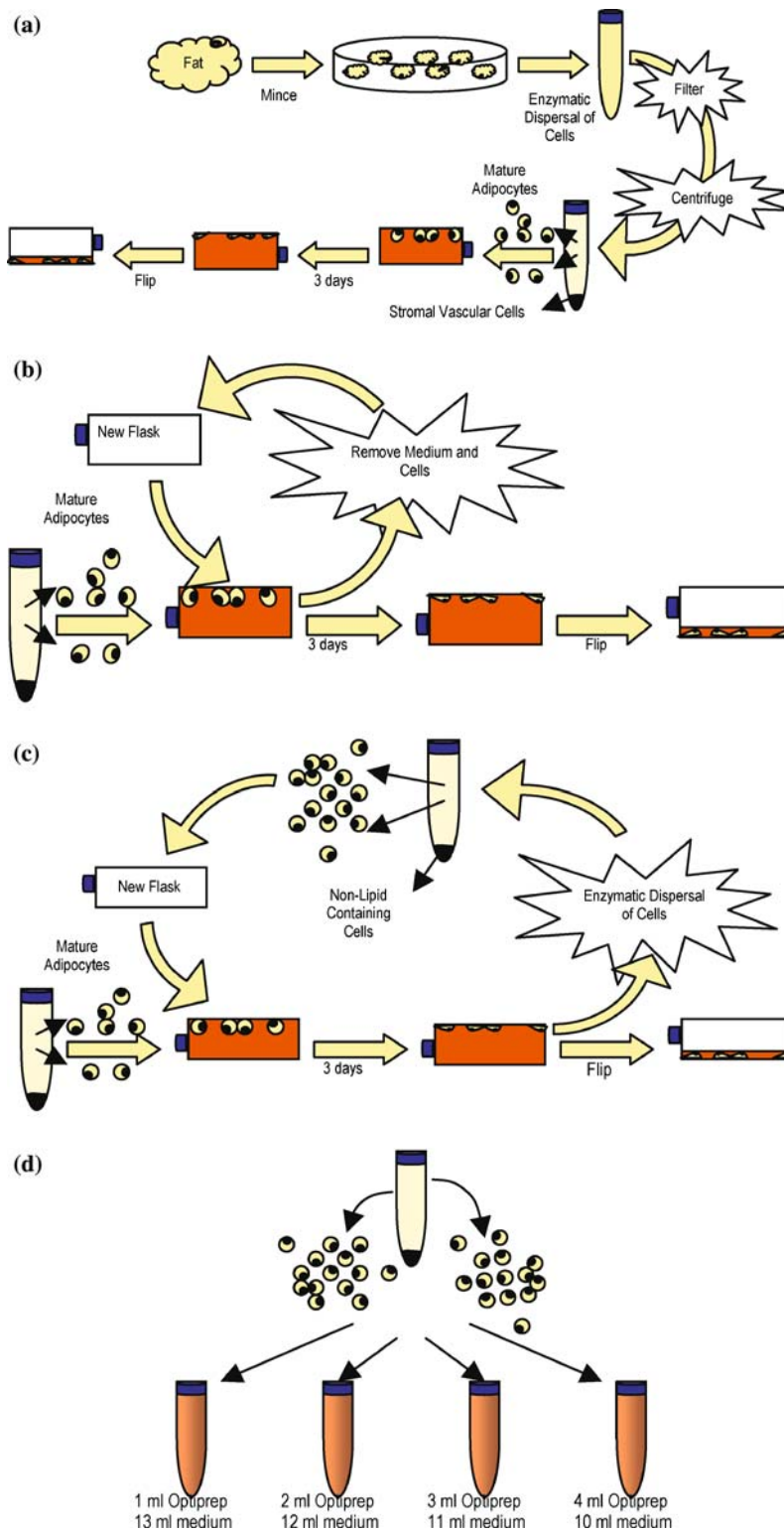


Figure 1. A flow diagram outlining the steps in (a) primary adipocyte isolation, (b) early differential plating, (c) late differential plating, and (d) isopycnic density gradient centrifugation.

removed with the medium) and the cells without lipid (preadipocytes and fibroblasts) to remain attached to the discarded flask.

Late differential plating

1. Enzymatically detach the cells from the culture flask by removing the medium from each flask and adding 2 ml of a 10% trypsin solution. Place the flask into the incubator.
2. After 4–5 min, monitor cell detachment under a phase contrast microscope. When the cells have all detached, remove the cell suspension, and pipette into a sterile 50 ml centrifuge tube.
3. Rinse the flask with 5 ml serum-containing medium and add to the 50 ml centrifuge tube.
4. Neutralize any remaining trypsin by adding 10 ml serum-containing medium to the 50 ml centrifuge tube.
5. Centrifuge for 10 min at $186\times g$.
6. Gently pipette the top 15 ml of supernatant into a clean 12.5 cm^2 flask and fill with DMEM/F12 + 10% HS.
7. Place the flasks in ceiling culture (i.e. invert the flasks). Cells that are lipid-laden will float and adhere to the culture surface whereas non-lipid containing cells, which had not previously discarded in the pellet, will sink and adhere to the non-culture surface. The cells usually attach within 24 h, after which time the excess medium can be removed and the flask re-inverted.
8. If, after the cells have attached, many non-lipid-containing cells are seen in the flask, a second differential plating may need to be performed to purify the cultures.

Isopycnic density gradient centrifugation

This method has been used for many years to separate not only different cell types from each other (especially in hematological studies) but also to separate intracellular organelles for experimentation. Herein, we describe a method to use Optiprep[®] to separate lipid-filled cells.

1. Pipette Optiprep[®] into a 15 ml centrifuge tube containing DMEM/F12 + 10% HS. Four different ratios are used in this experiment: 1 ml Optiprep[®] + 13 ml serum-containing medium, 2 ml Optiprep[®] + 12 ml serum-containing

medium, 3 ml Optiprep[®] + 11 ml serum-containing medium, and 4 ml Optiprep[®] + 10 ml serum-containing medium (Figure 1d).

2. Centrifuge the solution for 30 min at $800\times g$ to pre-form the gradient.
3. After the last step during initial adipocyte isolation (before placing in ceiling culture; Step 5), layer the uppermost 1 ml of supernatant on the preformed density gradient and centrifuge for 30 min at $800\times g$.
4. Once the centrifugation cycle is complete, transfer the upper 1 ml of supernatant to a flask and fill with a 1:1 mix of DMEM/F12 + 10% HS. Invert the flask and place in ceiling culture.

Cloning

The above methods will remove almost all of the non-lipid-containing cells from the cultures. Any remaining fibroblastic cells must be removed through culture surgery and cloning techniques.

1. After sufficient cell attachment in ceiling culture (1–5 d), followed by a primary method of purification, remove all but 5 ml of the medium from the flask.
2. Using a phase contrast inverted microscope, in a laminar flow hood mark the cells containing lipid with an indelible marker on the bottom of the flask for daily monitoring (Refer to Figure 3). Also, mark non lipid-containing cells for removal (with a non-permanent ink). Use different color inks for lipid-filled and non-lipid filled cells.
3. While looking through the microscope eye-pieces, scrape the cells previously marked for removal off the flask with a sterile Pasteur pipette.
4. Wash the cultures $3\times$ with DMEM + 10% HS and add 5 ml of medium to the flask.
5. Monitor the cultures daily and remove any non-lipid containing cells. The purified cultures are ready for experimentation.

Results and discussion

The methods describe here have been found to generate pure populations of mature adipocytes.

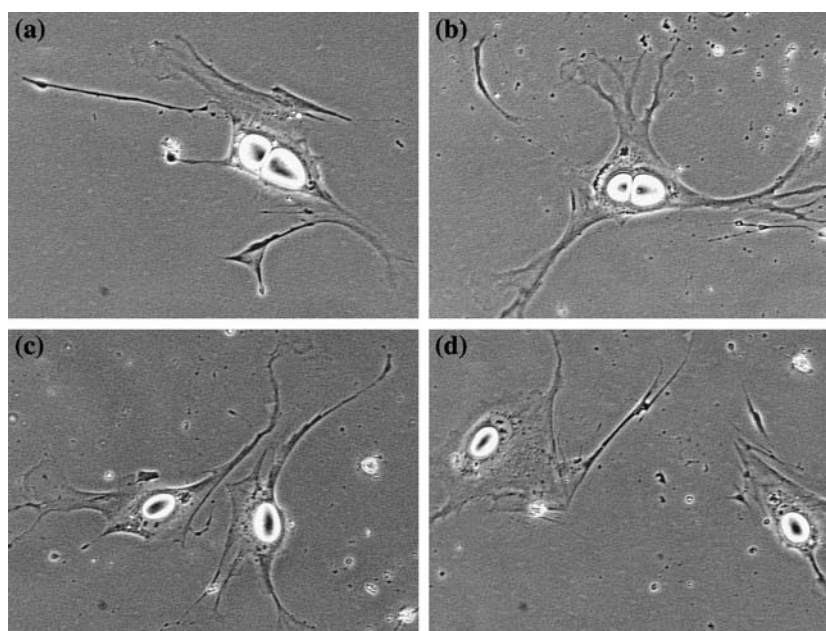


Figure 2. Photomicrograph of a bilocular bovine adipocyte undergoing proliferation *in vitro*. The culture was purified using the early differential plating protocol within 2 d of isolation and two serial late differential platings. Once the cultures were pure (no fibroblastic cells), the cultures were exposed to a traditional growth medium (DMEM + 10% FBS). The cultures were monitored daily for non-lipid laden cells and, once the culture was pure, the culture flask was exposed to a proliferative medium (DMEM + 10% FBS) for 5 d before cell division resulted: (a) the appearance of the maternal cell 24 h after the proliferative treatment was applied. Panel (b) shows the same cell after 3 d. Five days after the proliferative medium was applied (c), the cell divided, through symmetric cell division, into two daughter cells. Panel (d) shows the two daughter cells after 6 d. All photomicrographs were captured at 20 \times magnification with a NIKON inverted Diaphot microscope, equipped with Sony RGB (0.6 in chip) camera and OPTIX image analysis system.

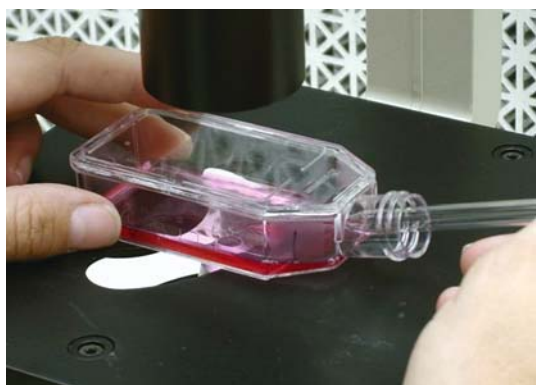


Figure 3. Photograph of a flask during the cloning procedure. The microscope has been placed in a laminar flow hood and the non-lipid containing cells have been marked on the bottom of the flask. In the hood, the cap was removed from the flask and the Pasteur pipette was inserted into the flask. While looking through the eyepieces of the microscope, the marked cells are scraped off the plate.

Although this laboratory has employed all the described techniques in various different combinations, the cells displayed in Figure 2 were obtained with an early differential plating 2 d after first isolation and two sequential late differential platings. After the cultures were purified (i.e. without any fibroblastic/preadipocyte cells) the cultures were exposed to a traditional growth medium (DMEM + 10% FBS). The cells were monitored daily for proliferative activity, which subsequently occurred. In fact, a previous communication (Fernyhough et al., 2005b) showed mitotic figures in mature adipocytes just prior to mitosis. These cells may be studied for regulation of lipolysis as well as for mechanisms underlying their propensity to proceed from the mature phenotype to a proliferative-competent (presumably less differentiated) cell type (preadipocyte or adipofibroblast – termed reverse differentiation or dedifferentiation). In

Table 1. Troubleshooting table.

Problem	Solution
Low cell yield from isolation	<p>(1) Shorten the time from removal of tissue to processing. Keep the HBSS solution at 37 °C</p> <p>(2) When removing the tissue from the enzyme solution check the turbidity of the liquid. If it is clear or only slightly turbid, you may want to increase the incubation time.</p> <p>(3) Leaving the tissue in the enzyme solution for too long will have a detrimental effect on the cells – shorten the incubation time. (4) If the collagenase is out dated or appears to have lost activity a replacement with fresh collagenase is warranted. (5) Remove more of the supernatant to place in ceiling culture</p> <p>(1) Increase the centrifugation time during initial isolation to pellet the heavier non lipid-containing cells. (2) Perform an early differential plating.</p> <p>(1) Check the centrifuge settings. (2) Increase the amount of centrifuge time to allow the heavier mononucleated cells to pellet at the bottom of the tube. (3) Remove less of the supernatant taking care to remove the uppermost supernatant. (4) Perform a second late differential plating.</p> <p>(1) Check the centrifuge settings. (2) Decrease the amount of centrifuge time allowing the lipid-laden cells to remain suspended. This solution may yield more fibroblastic cells than desired. Perform a second differential plating</p> <p>(1) With a small number of fibroblastic cells on the culture surface, it is easy to perform cloning techniques. Alternatively (2) another late differential plating may be performed.</p> <p>(1) Check the centrifuge settings. (2) Increase the spin time when pre-forming gradient. (3) Increase the amount of Optiprep[®] placed in culture after the final spin</p>
This can occur from a number of problems: (1) the time from removal of tissue from animal to processing is too long; (2) the tissue was left in the enzyme solution for too long or (3) not long enough; (4) the collagenase used in the enzyme solution has lost its activity; or (5) not enough of the supernatant was removed during the last step in isolation	
Large numbers of mononucleated cells in the flask after isolation	
Large numbers of mononucleated cells in the flask after late differential plating	
Low adipocyte cell yield from late differential platings	
Spinning too fast or for too long will disrupt the membranes of the lipid-laden cells causing a low cell yield	
Low numbers of fibroblastic cells in the cultures after late differential plating	
Low adipocyte cell yield from isopycnic density gradient centrifugation	

addition, the progeny cells are homologous and may be used for detailed study of the regulation of lipogenesis and dynamics of lipid accumulation.

The photomicrographic results obtained through these procedures clearly demonstrate the ability of these mature adipocytes to proliferate *in vitro* (Figure 2; Dodson et al. 2005; Fernyhough et al. 2005a, b). In this communication, we have presented additional evidence that mature adipocytes proliferate *in vitro*. This concept has previously been disputed, stemming from the idea that once the cells accumulated intracellular lipid, adipocytes were considered 'terminally differentiated.' Our overall hypothesis is that the accumulation of lipid is not a terminal event for these cells. Our next set of objectives include: (a) attempting to determine the specific number of cells, in any adipose depot, that are capable of dedifferentiating, (b) Evaluating this phenomenon in young vs. old animals, animals of different sexes and breeds, as well as (c) other physiological states that may influence the dedifferentiation process. As we have not defined the specific cellular and biochemical/molecular characteristics of the cells that are capable of dividing, it may be possible that the isolated lipid-filled cells in Figure 2 represent a different cell form, such as a fibroblast, which may accumulate and release lipid in a similar manner to an adipocyte. In addition, other, yet unknown, cells, that are capable of metabolizing lipid similar to adipocytes, might also reside in these depots. However, we suggest that this area of research is of sufficient interest such that a re-evaluation of the cell composition of adipose tissue depots may be needed. Collectively, this research area may lead to a new understanding of adipose tissue growth and development.

Application of methods

The many processes regulating adipose tissue development are of considerable interest due not only to their physiological effects, but also to their potential effects on current medical concerns. For example, obesity is of great concern as adults classified as overweight topped 64.5% (Flegal et al. 2002) and has been deemed a predisposing factor in other medical conditions such as diabetes and heart disease. Indeed, both diabetes and hypertension has been steadily increasing every

year in the U.S. The percentage of adults who have been ever been diagnosed as having diabetes was 6.6% in 2003 (up from 5.1% in 1997; Schiller et al. 2005) and the percent of adults with hypertension from 1999 to 2002 was 30.1% (Schiller et al. 2005).

In addition to the biomedical field, adipose development is of concern in the economically important traits of food-producing animals such as cattle. These traits include fat depth and distribution (including marbling) as well as the overall efficiency of nutrient use (Hansen et al. 2004). For example, a reduction in subcutaneous fat (waste fat) would be of tremendous economic savings to producers. The total cost of excess fat to the U.S. beef industry has been shown to be \$ 4.4 billion; \$ 2 billion in production costs and \$ 2.4 in shipping and removal costs (Ritchie et al. 1993). Additionally, marbling has been shown to influence consumer preferences for beef (meat) products. Understanding the mechanisms underlying adipocyte differentiation and adipogenesis could eventually help producers to modulate the characteristics of adipose tissues and promote the occurrence of marbling deposition without the excess accumulation of subcutaneous or visceral fat (Hansen et al. 2004).

Acknowledgements

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