HHS Public Access

Author manuscript

Methods Mol Biol. Author manuscript; available in PMC 2018 March 27.

Published in final edited form as:

Methods Mol Biol. 2017 ; 1566: 77–85. doi:10.1007/978-1-4939-6820-6_8.

Protocols for generation of immortalized human brown and white preadipocyte cell lines

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Abstract

Human brown and white preadipocytes offer a unique cell model to study human adipogenesis and thermogenesis. Here, we describe the detailed procedures for isolation of human brown and white predipocytes from deep and superficial neck fat. To grow these cells *in vitro* for a prolonged period of time, they should be immortalized following the procedure discussed here. We also provide the protocol for expansion, cryopreservation, and adipogenic differentiation of cells.

Keywords

Brown and white adipose tissue; Stromal Vascular fraction (SVF); Preadipocytes; neck fat; Immortalization; Differentiation

1. Introduction

Human obesity results from an imbalance between energy intake and expenditure, and it is a major contributor to metabolic syndrome and disorders, such as type 2 diabetes, cardiovascular disease, and some cancers. Brown and white adipose tissue are two functionally distinct types of fat present in mammals. While the main role of white adipose tissue (WAT) is storing excess calories, brown adipose tissue (BAT) specializes in energy dissipation and thermogenesis through the activity of uncoupling protein 1 (UCP1). BAT can be activated by cold stimulation in both humans and rodents. Cold-activated BAT in humans consume more glucose per tissue weight than any other tissue (1). Given the ability of BAT to utilize fuel and transform chemical energy of nutrients into heat, increasing the activity or amount of human brown fat constitutes a promising strategy to combat obesity.

The recent rediscovery of functional BAT in adult humans was made by using 18F-fluorodeoxyglucose positron emission tomography/computed tomography (18F-FDG PET/CT) as bilaterally symmetric patches of radio-labeled glucose uptake in the neck and supraclavicular, region (2). Later on, UCP1-expressing adipocytes were reported to exist in adult humans around the neck, supraclavicular and spinal cord regions (3–5). Most importantly, the amount of brown adipose tissue was shown to be inversely correlated with

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body-mass index and percentage of body fat, whereas positively correlated with resting metabolic rate in human subjects.

Examination of adipose tissue collected from different depots of the human neck revealed that superficial and deeper fat had the classical histological, ultrastructural, and gene expression feature of rodent white and brown adipose tissue, respectively. Importantly, deeper human neck fat expressed higher levels of UCP1 compared to the more superficial neck depots (6). To enable comprehensive studies of molecular and cellular aspects of human adipogenesis and thermogenesis *in vitro*, we have established pairs of immortalized human brown and white preadipocytes from human neck fat biopsies. Procedures of generation, growth, and differentiation of these cells are detailed below.

2. Materials

2.1. Equipment

1. Laminar flow hood
2. Humidified CO2 incubator
3. Water bath
4. Centrifuge
5. pH meter
6. Chemical balance
7. Stirrer
8. Cell Culture vessels
9. Serological pipettes
10. Aspiration pipettes
11. Inverted microscope
12. "Mr. Frosty" freezing container
13. Liquid Nitrogen tank
14. Hemocytometer or automated cell counter
15. 100 μm Cell Strainer
16. Parafilm
17. Sterile dissection instruments

2.2. Reagents

1. Digestion solution: 2mg/ml Collagenase-I in 3.55 BSA in PBS. Dissolve 20 mg Collagenase-I in 10 ml PBS buffer. Add BSA to final concentration of 3.5% to the solution. Filter using a 0.22 μM filter unit. Prepare approximately 10 ml per sample.

2. Growth medium: 10% FBS in DMEM/High glucose. Add 100 ml Fetal Bovine Serum (FBS) to 890 ml DMEM/High Glucose. Add 10 ml Penicillin-Streptomycin. pH to 7.4 using either HCl or NaOH and sterile filter. For preparation of growth medium with gentamicin, add 1000× stock solution to growth medium and sterile filter.
3. Freezing medium: 50% FBS and 10% DMSO in DMEM/High glucose. Add 25 ml FBS to 20 ml DMEM/High Glucose. Add 5 ml DMSO. Sterile filter.
4. Differentiation and Induction medium: Prepare the reagent stocks for differentiation and induction medium as follow: Biotin: dissolve 0.08g in 10ml 0.1N NaOH to make 30 mM solution. Insulin: dissolve Insluin in cncentration of 10 mg/ml in 0.01 M HCl. Pantothenate: dissolve 8.5 mM solution in H2O (500× stock). Dexamethasone: dissolve 0.002g in 1ml EtOH (2mg/ml; 5 mM). Isobutyl methylxanthine (IBMX): make 50 mM stock in 0.1 M KOH. 3,3′,5-Triiodo-L-thyronine (T_3): add 1.0 ml of 1.0 N NaOH to 1 mg, gently swirl to dissolve the powder and add 152.6 ml of sterile medium (10 μM). Indomethacin: Dissolve 447.25 mg Indomethacin in 10 ml EtOH (0.125 M). Heat to 75°C. Aliquot stock solutions and store them at −20°C. Avoid multiple freeze/thaw cycles. Differentiation Medium: Add 1 ml FBS to 48.5 ml DMEM/High Glucose. Add 500 μl Penicillin-Streptomycin, 15 μl human Insulin (0.5 μM), and 10 μl T3 (2nM). pH to 7.4 using either HCl or NaOH and sterile filter. Induction Medium: Add 1 ml FBS to 48.5 ml DMEM/High Glucose. Add 500 μl Penicillin-Streptomycin, 50 μl Biotin (33 μM), 15 μl human Insulin (0.5 μM), 100 μl Pantothenate (17 μM), 1 μl Dexamethasone (0.1 μM), 10 μl T3 (2nM), 500 μl IBMX (500 μM), and 12.5 μl Indomethacin (30 μM). pH to 7.4 using either HCl or NaOH and sterile filter.
5. Trypsin-EDTA (0.25%)
6. PolyJet In Vitro DNA Transfection Reagent
7. pBate-hTert-hygro or pBate-hTert-neo retroviral plasmids (Addgene, Cambridge, MA)

3. Methods

3.1. Isolation of brown and white fat progenitors from human neck fat

All the steps should be carried out under a laminar flow hood.

1. Remove fat sample from collection tube and transfer onto a piece of parafilm.
2. Add a small amount of digestion solution to the tissue to avoid drying.
3. Mince the tissue in the solution to a fine consistency.
4. Transfer to a 50 ml tube containing 10 ml digestion solution and seal the tube with parafilm. For isolation of human BAT progenitors (hBAT-SVF cells), tissues collected from deep neck fat (carotid sheath, longus colli, and prevertebral depots) should be combined. For isolation of human WAT progenitors (hWAT-

SVF cells), tissues collected from superficial neck fat (subcutaneous and subplatysmal depots) were combined.

5. Vortex each tube thoroughly and put the tubes in the water bath to shake at 70 rpm and 37 °C. Make sure that the water bath is full enough so that the tissue is submerged.
6. Vortex the tubes thoroughly every 10 minutes. The tubes should remain in the bath until all lumps appear dissolved, but not so long that a clear fat supernatant layer appears (up to 45 minutes – longer digests tend to result in more cells, but over-digestion can decrease the cell yield).
7. When digestion is complete, add 3 ml FBS to stop Collagenase and pipet thoroughly.
8. Pass through a 100 um filter into a fresh 50 ml tube (wash the tube again with 10 ml growth medium, then filter).
9. Centrifuge the tubes at 300 g for 10 minutes.
10. Gently aspirate off most of the supernatant and resuspend the pellet in 10 ml growth medium.
11. Centrifuge at 300 g for 10 minutes.
12. Gently aspirate off the most of the supernatant leaving only the pellet.
13. Resuspend the pellet in 10 ml growth medium with gentamicin.
14. Centrifuge at 300 g for 10 minutes.
15. Gently aspirate off the most of the supernatant and repeat wash/spinning again.
16. Gently aspirate off the most of the supernatant.
17. Resuspend the pellet in 1 ml growth medium with gentamicin.
18. Seed the cells in growth medium in one well of a 12-well plate. Add 1 ml growth medium to wash the tube again, then transfer to the same well.
19. The next day, carefully wash the cells only one time with growth medium +gentamicin (some cells might be loosely attached to the plate, make sure not to detach them while washing).
20. 48 hours later, repeat washing.
21. Remove Gentamicin from the growth medium after 72 hours.

3.2. Generation of immortalized human brown and white fat progenitors

Preparing retrovirus using amphotropic Phoenix (Phoenix-A) cell line:

In this protocol, a retroviral plasmid (pBate-hTert-hygro or pBate-hTert-neo) is used to deliver human Telomerase reverse transcriptase (hTERT) to primary hBATand hWAT-SVF cells. For production of high titer retrovirus for infection, helper free Amphotropic Phoenix (Phoenix-A) cell line can be used. Phoenix Ampho and Eco

packaging cell lines are second-generation retrovirus producer lines for the generation of helper free amphotropic and ecotropic retroviruses. Phoenix-A Cell Line can be used to deliver genes to dividing cells of most mammalian species, including human (see note 4.1) (7,8).

Day 0: Preparation of Phoenix-A Retrovirus Producer cells for Transfection

1. 18–24 hours prior to transfection, seed Phoenix-A cells at 4–5 million cells per 10-cm plate in growth medium.
2. After plating cells, transfer plates to the incubator. Do not disturb the cells for several hours to ensure their attachment.

Day 1: Transfection

3. One hour prior to transfection, change the medium of Phoenix-A cells and add 6 ml fresh growth medium to each 10-cm plate. Be careful not to detach the cells.
4. For each 10-cm plate, dilute 5 μg of DNA into 250 ul of serum-free DMEM with High Glucose. Gently pipette up and down or vortex briefly to mix.
5. For each 10-cm plate, dilute 15 μl of PolyJet™ reagent into 250 μl of serum-free DMEM with High Glucose. Pipette up and down 3–4 times to mix.
6. Immediately add the diluted PolyJet™ reagent solution to the diluted DNA. Pipette up and down 3–4 times or vortex briefly to mix. Let the mixture to stand for 10–15 minutes at room temperature to allow DNA/reagent complexes to form.
7. Add the 500 μl DNA/reagent mixture to the plate in a drop-wise manner and homogenize the mixture by gently swirling the plate.
8. 6–12 hours post infection, remove medium containing DNA/reagent mixture and add fresh growth medium to the cells.

Day 2: Preparation of primary hBAT-SVF and hWAT-SVF cells for infection

9. Plate 4–8×10^4 cells in one well of a 12-well plate. To achieve maximum infection efficiency, cells should be around 60–80% confluent. Make sure to include one control well for drug selection.

Day 3: Collection of retrovirus particles and infection of primary hBAT-SVF and hWAT-SVF cells

10. Harvest the Phoenix-A cells supernatant containing retrovirus particles.
11. Filter through 0.45 um filter to removes cells in the suspension.
12. Virus can be used freshly for infecting the target cells. Alternatively, aliquot the virus and immediately freeze at −80 C for later infection. Avoid freezing and thawing of virus as it decreases virus titer (see note 4.2).
13. For infection of hBAT-SVF and hWAT-SVF cells, dilute pBate-hTert-hygro virus in growth medium in 1:1 ratio. Add polybrene to final concentration of 4ug/ml. Add the mixture to cells.

14 Repeat step 4. every day while the cells are growing until the cells reach near 90% confluency (usually 3 times).

15 Once cells are around 90% confluent, split the cells in each well into two wells.

16 The next day, start drug selection with the proper selectable marker. If using hygromycin, start with 100 ug/ml hygromycin in growth medium. For Neomycin selection, start with 500 ug/ml G418 (Geneticin). The optiomal concentration of antibiotic can vary between subjects, and should be determined for each cell line (see note 4.3).

17 Closely monitor cells' response to the selection drug. A large number of control cells should die within 2–3 days. If little or no cell death is observed, increase drug concentration. To ensure effective selection, change medium containing drug every other day.

18 Once all control cells (no infection) die, stop selection and add growth medium containing low concentration of antibiotic (50 ug/ml Hygromycin or 200 ug/ml G418) for one more week.

19 Remove the antibiotic and let the cells grow without antibiotic for several days. Immortalized human preadipocytes will start to proliferate and clusters of cells will appear after almost a week.

20 Once they are 80–90% confluent, split the cells or freeze them according to the freezing protocol described below.

3.3. Maintenance and expansion of immortalized human preadipocytes

Grow the cells in growth medium at 37°C in a humidified incubator with 5% CO2.

When cells reach 80–90% confluency, split them 1:2 or 1:3 (see note 4.4 and 4.6).

To subculture, aspirate culture medium. Rinse once with PBS (Calcium and Magnesium free). For a 10 cm dish, add 1mL of 0.25% (w/v) pre-warmed trypsin and return to incubator for 2–3 minutes. Observe cells under an inverted microscope. When cells start to become round and detach from the plate, neutralize trypsin by adding 10mL of growth medium to the dish and collect cells by gentle pipetting. Plate the cells in new dishes and incubate in the incubator and let them adhere.

Change culture media the following day to remove non-adherent cells.

3.4. Cryopreservation of primary and immortalized human preadipocytes

1. Prepare freezing medium as described above.
2. Gently detach cells from the tissue culture vessel following the procedure described above for subculture.
3. After adding growth media and collecting cells by pipetting, transfer cell solution to a 15 ml tube and centrifuge at 300 g for 4 minutes.
4. Gently remove the tube from centrifuge, aspirate most of the supernatant without disturbing the cell pellet, and resuspend pellet in 1 ml freezing media per vial to

be frozen. To ensure maximum recovery of frozen cells, each confluent 10-cm dish should be frozen in two vials.

5. Place vials in "Mr. Frosty" freezing container containing100% isopropyl alcohol at −80 °C for 24 hours.

6. Transfer vials to liquid N_2 tank for indefinite storage.

3.5. Differentiation of immortalized human white and brown preadipocytes

1. Seed 40,000–50,000 cells per well in a 24-well plate.

2. Let the cells grow in growth medium until they are fully confluent (Usually 3–4 days for most cell lines, up to 6 days) (see note 4.5).

3. Once cells reach confluency, aspirate growth media and add freshly prepared induction medium to cells.

4. Add fresh induction media every three days for 12 days.

5. Harvest differentiated adipocytes for further analysis (Gene expression analysis, Oil red O staining, etc.)

6. For some lines, Rosiglitazone (1 μM) should be added in the induction medium to enhance differentiation.

Alternatively, to induce increased *UCP1* expression, mitochondrial activity and fuel utilization in mature adipocytes, cells can be pretreated with BMP7 in differentiation medium prior to adipogenic differentiation as described below (9–11).

1. Add 3.3 nM BMP7 or similar volume of vehicle to the differentiation medium.

2. Once cells reach confluency, aspirate growth media and add differentiation medium containing BMP7 or vehicle to cells.

3. Treat cells with BMP7 in differentiation medium for six days. Add fresh differentiation medium every three days.

4. At day 7, start adding induction media to cells.

5. Change induction medium every three days for 12 days.

4. Notes

4.1. Handling Phoenix cells

Never let Phoenix-A cells reach confluence, as it decreases their transfection efficiency. For maximally healthy culture condition, passage cells when they are 70–80% confluent in 1:4 or 1:5 ratios.

4.2. Virus concentration

To increase the titer of retrovirus preparations, supernatant can be concentrated using commercially available retrovirus concentration kits or other in-house methods. Try to use fresh virus when possible, as the titer decreases with each freeze-thaw cycle.

4.3. Drug selection

Cells derived from different human subjects can vary in their response to Neomycin or Hygromycin. Try different concentrations for each cell line. Monitor the infected and non-infected control cells closely while they are under drug selection.

4.4. Maintenance of immortalized human white and brown preadipocytes

To ensure an optimal growth condition, never plate the cells too thin. Some cell lines tend to grow very slowly when they are below 20–30% confluent. Splitting 80–90% confluent culture in 1:2 to 1:3 ratios usually provides a good starting confluency.

4.5. Differentiation of immortalized human white and brown preadipocytes

Never start adding the induction medium to cells before they are completely confluent. Sub-confluent preadipocytes do not differentiate efficiently. The optimal starting cell number can vary slightly between cell lines and it should be determined for each individual cell line.

4.6. Mycoplasma contamination

Test the cells for mycoplasma contamination on a regular basis. Stick to good aseptic technique to prevent contamination. Treat contaminated cells with an antimycoplasma reagent such as Plasmocin™ (InvivoGen, San Diego, CA) according to manufacture's instruction.

Acknowledgments

This work was supported in part by NIH grants R01 DK077097 and R01 DK102898 (to Y.-H.T.), and P30 DK036836 (to Joslin Diabetes Center's Diabetes Research Center).

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