Efficient differentiation of beige adipocytes from adult human adipose-derived stem/stromal cells

Amar M. Singh
University of Georgia
Liang Zhang
University of Georgia
John Avery
University of Georgia
Stephen Dalton (sdalton@uga.edu)
University of Georgia https://orcid.org/0000-0002-3450-1263

Method Article

Keywords: beige adipocytes, pluripotent stem cells, diabetes

Posted Date: August 1st, 2020

DOI: https://doi.org/10.21203/rs.3.pex-888/v1

Abstract

Beige adipocytes (also known as brite adipocytes) have significant utility for numerous applications, such as drug screening, cell therapy, and disease modelling. However, a high-efficiency protocol from human adult adipose-derived stem/stromal stem cells (ADSC) has not been described. The protocol described here achieves beige adipocyte purities of >92% in a fully-defined, serum-free media cocktail, which enables these downstream applications. This method provides a significant leap forward over previously described, serum-based protocols that were inconsistent and inefficient.

Introduction

Thermogenic adipocytes (brown adipocytes and beige/brite adipocytes) have emerged as being a therapeutically useful cell type for the treatment of obesity-associated metabolic diseases such as type 2 diabetes[1, 2]. While brown adipocytes are derived through a paraxial mesoderm-dermomyotome precursor, beige/brite adipocytes are generated from an adult adipose progenitor cell[1, 2]. Unlike traditional white adipose cells that store lipids, thermogenic adipose cells uptake excess fatty acids and glucose from the bloodstream so that they can burn it and release it as heat. This process enables thermogenic adipocytes to normalize whole-body glucose levels and reduce body weight[1, 2]. For these reasons, thermogenic adipocytes are thought to have therapeutic utility[1-3]. While methods have been developed to generate beige/brite using rodent cells or immortalized human lines, protocols have not been established to generate these cells from human adult ADSC with high purity and efficiency[3].

In the associated publication, the protocol described here was compared to two previously described methods[4, 5]. Both of these methods relied on serum-based approaches that resulted in inconsistent differentiation efficiencies. The UCP1 levels observed in this protocol were consistently higher at both the transcript and protein level than the methods described by Bartesaghi[4] et al. or Wang[5] et al. The beige adipocytes developed by this protocol were also validated functionally in vivo following transplantation by indirect calorimetry, along with glucose measurements in diabetic models and histological analysis. The beige adipocytes were also used for high throughput drug screens demonstrating the robustness of the differentiation platform.

In total, 6 primary ADSC lines (purchased from ThermoFisher or Lonza) have been evaluated using this protocol with varying sex, BMI and diabetic status, and indicated efficient differentiation based on UCP1 staining. Major differences could not be noted between any of these factors. Here a protocol is provided using the StemProÔ Human Adipose-Derived Stem Cell Kit for differentiation to beige adipocytes. As an alternative choice, the Lonza ADSC, along with the ADSC Growth Medium BulletKitÔ may be used.

Reagents

- Albumin, Bovine (EMD Millipore, 821005)
- Antibiotic-antimycotic (Fisher Scientific, MT30004CI)
- Nonessential amino acids (Fisher Scientific, MT25025CI)
- GlutaGro (Fisher Scientific, MT25015CI)
- Trace Elements A (Fisher Scientific, MT99182CI)
- Trace Elements B (Fisher Scientific, MT99175CI)
- Trace Elements C (Fisher Scientific, MT99176CI)
- Sodium L-ascorbate (Sigma, A4034)
- Transferrin (Athens Research Technology, 16-16-032001-LEL)
- DMEM/F-12 (Fisher Scientific, MT15090CM)
- BMP7 (Biotechne, 354-BP/CF)
- IGF1 (Sigma/SAFC, 85580C)
- FGF2 (Biotechne, 4114-TC)
- Y27632 (Biotechne, 1254)
- Rosiglitazone (Biotechne, 5325)
- Dexamethasone (Biotechne, 1126)
- Triiodo-L-thyronine (Sigma, 64245)
- IBMX (Cayman Chemicals, 13347)
- StemProÔ Human Adipose-Derived Stem Cell Kit (ThermoFisher, R7788110)
- Optional: Lonza hADSC (Lonza, PT-5006); Bulletkit Growth medium (Lonza, PT-4505)
- TrypLE Express, no phenol red (ThermoFisher, 12604013)
- Dulbecco’s PBS without calcium and magnesium (Fisher Scientific, MT21031CM)
- Fetal Bovine Serum, stem cell qualified (Atlanta Biologicals, S10250)
- Forskolin (Biotechne, 1099)

· Optional: Stempro Adipogenesis Differentiation Kit (Thermofisher, A1007001)

Equipment

· Laminar flow hood

· CO2 incubator

· Microscope (eg. Leica MZ16)

· Swinging bucket cell culture centrifuge (eg. Accuspin 1R)

· Liquid nitrogen tank

· Tissue culture dishes, 100mm (Fisher Scientific, 12-556-002)

· Tissue culture plates, 6-well (Fisher Scientific, 08-772-1B)

· Stericup-GP 0.22 mm vacuum filtration flask, 500ml (Millipore, SCGPU05RE)

· Conical centrifuge tubes, 15 ml (Corning, 430790)

· Plastic pipettes (5, 10, 25 and 50 ml)

· Hemocytometer or other cell counting device

· Cryogenic tubes (NUNC, 377267)

· Freezing container (Sigma, CLS432000)

Procedure

A. Thawing, expansion and freezing of Adipose-derived stem/stromal cells (ADSC)

1. Refer to the user guide for StemProÔ Human Adipose-Derived Stem Cell Kit (ThermoFisher, R7788110), from here on referred to as the “StemProÔ user guide”.

2. Prepare the complete MesenPRO RS medium, by adding 10 ml of RS growth supplement and 5 ml of glutamine solution according to the manual. In addition, add 5 ml of antibiotic-antimycotic solution and filter sterilize through a 500 ml Stericup filtration flask.

3. Refer to StemProÔ user guide and follow the procedure for thawing ADSC.

4. Change media on ADSC every 3 days using MesenPRO RS complete medium.

5. Passage and expand ADSC once the cells have become ~90% confluent using TrypLE Express, according to the StemProÔ user guide. **Do not allow ADSC to become 100% confluent when expanding cells.**

6. Continue to expand cells until there are ~10 100mm dishes, which typically yields 1-1.5 million cells per plate.

7. Prepare freezing solution according to StemProÔ user guide, which consists of 20% fetal bovine serum, 10% DMSO in MesenPRO RS complete medium.

8. Freeze ~10 vials of ADSCs with 1 million cells per vial, according to the StemProÔ user guide.

B. Generation of Beiging-8 (B-8) medium

1. The first step of generating the B-8 medium is developing the defined medium (DM). Into a 500 ml Stericup add the following to generate the DM:

· 50 ml of Albumin (20%, vol/vol in DMEM/F-12)

· 5 ml of antibiotic-antimycotic solution (100X)

· 5 ml of glutaGRO (100X)

· 5 ml of minimal essential amino acids (100X)

· 0.5 ml of Trace Elements A, B and C

· sodium ascorbate (50 mg/ml, final concentration)

· transferrin (10 mg/ml, final concentration)

· 432.75 ml of DMEM/F-12

· Filter-sterilized the DM.

-

2. Aliquot and heat 5 ml of DM in a 15 ml conical tuble, along with a 500 ml aliquot of IBMX (500 mM), to 65°C for 15 minutes.

3. To the remaining DM add the following to generate B-8:

· IGF-1 (200 ng/ml, final concentration; stock: 1 mg/ml)

· FGF2 (8 ng/ml, final concentration; stock 25 mg/ml)

· BMP7 (100 ng/ml, final concentration; stock 100 ug/ml)

· Y27632 (10 mM, final concentration; stock 10 mM)

· Rosiglitazone (2 mM, final concentration; stock 20 mM)

· Dexamethasone (1 mM, final concentration; stock 10 mM)

· Triiodo-L-thyronine (1 nM, final concentration; stock 20 mM)

· Once the IBMX (500 mM, final concentration; stock 500 mM) is heated and fully re-suspended, **rapidly** add it to the 5 ml of heated DM. This may then be added to the remaining DM—which will generate the complete B-8 medium.

· Medium may be stored at 4°C for up to 2 weeks.

C. Differentiation of ADSC to beige adipocytes.

1. Thaw an aliquot of ADSC as described in the StemProÔ user guide, and expand the cells as needed for planned downstream applications. Cells may be passaged for ~10 times without major cell senescence (a 1:4 or 1:5 split may be used for routine passaging).

2. Plate ADSC into each well of a 6-well plate (5000 cells/cm^2) using MesenPRO RS complete medium and change medium every 3 days.

Optional: a 2nd plate of ADSC can be generated to serve as an ADSC control plate or to generate white adipocytes using the Stempro adipogenesis differentiation kit (A1007001).

3. **Grow ADSC until they reach 100% confluency prior to differentiation induction**. **This is a critical step for efficient differentiation.**

4. Pre-warm an aliquot of B-8 media in a 37°C water bath.

5. Aspirate media from 6-well plate and wash cells 2X's in DPBS to remove any residual media.

6. Add 2 ml of pre-warmed B-8 media to each well of the 6-well plate containing confluent ADSCs. This is the starting point (day 0) for the differentiation to beige adipocytes.

The ADSC from the control plate may be imaged and collected for RNA/protein analysis or used to generate white adipocytes beginning at this time point.

7. The media on the beige adipocyte plate should be changed every 48 hours for three weeks.

8. On day 20-21, Forskolin (20 mM, final concentration) may be added to the beige cells for 24 hours for protein or 6 hours for RNA.

9. Beige adipocytes can then be collected in triplicate for RNA/protein analysis or other downstream applications using conventional approaches.

Anticipated results

1. Initial differentiation of ADSC to beige cells may have the appearance of apoptotic cells or undergoing cell death. However, by days 5-6, small lipid-filled vesicles should become apparent in the cells.

2. By day 21, cell culture should be strikingly different from ADSC with uniform multiloculular lipid droplets. Stopping cultures early (~day 15) results in significantly reduced levels of *UCP1*.

3. Transcript markers most useful for evaluation include *UCP1*, *DIO2*, *ADIPOQ and PPARGC1*. These markers are not detectable in ADSC but will be significantly elevated upon differentiation to beige adipocytes. Forskolin will result in further a 2-3 fold induction of *UCP1, and a >5-fold induction in DIO2*.

4. Immunostaining of the cultures will reveal that >90% are positive for UCP1.

Troubleshooting

1. Low cells survival upon differentiation.

a. Confirm that you are using a high grade of bovine albumin suitable for cell culture. Low quality grades, such as BSA-fraction V is not suitable.

b. Confirm that media is freshly made and that growth factors have not underwent freeze-thaw cycles.

c. Confirm there are no sources of contamination in the cells, such as mycoplasma.

2. Differentiation efficiency to UCP1-expressing beige adipocytes is low.

a. Confirm that cells are grown to 100% confluency.

b. Passage ADSC 5-6 times prior to beginning differentiation, as later passaged cells appear to have enhanced differentiation efficiencies.

c. While all lots tested so far work very well, some lot-to-lot variability may be expected, as with all cell lines.

Time Taken

1. ADSC thawing, passaging and expansion: 3 weeks
2. B-8 media preparation: 30-45 minutes
3. Beige adipocyte differentiation: 3 weeks

Anticipated Results

This protocol will generate >90+ UCP1+ beige adipocytes.

References

1. Harms, M. & Seale, P. Brown and beige fat: development, function and therapeutic potential. *Nat Med* **19**, 1252-1263 (2013).

2. Lidell, M.E., Betz, M.J. & Enerback, S. Brown adipose tissue and its therapeutic potential. *J Intern Med* **276**, 364-377 (2014).

3. Singh, A.M. & Dalton, S. What Can 'Brown-ing' Do For You? *Trends Endocrinol Metab* **29**, 349-359 (2018).

4. Bartesaghi, S. et al. Thermogenic activity of UCP1 in human white fat-derived beige adipocytes. *Mol Endocrinol* **29**, 130-139 (2015).

5. Wang, Y.L., Lin, S.P., Hsieh, P.C. & Hung, S.C. Concomitant beige adipocyte differentiation upon induction of mesenchymal stem cells into brown adipocytes. *Biochem Biophys Res Commun* **478**, 689-695 (2016).

Acknowledgements

This work was funded by a Georgia Research Alliance Ventures Grant to S.D.