A protocol for the differentiation of brown adipose progenitors derived from human induced pluripotent stem cells at a high efficiency with no gene transfer

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Method Article

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Abstract

Human induced pluripotent stem cells \(hiPSC) show great promise for obesity treatment as they represent an unlimited source of brown/brite adipose progenitors \(BAPs). However, BAPs derived from hiPSCs \(hiPSC-BAPs) display a low adipogenic capacity compared to adult-BAPs when maintained in a traditional adipogenic cocktail. The reasons of this features are unknown and hamper their use both in cell based therapy and basic research. We describe the steps in details to derive BAPs from hiPSCs and to differentiate hiPSC-BAPs at a high rate into mature brown adipocytes. hiPSC-BAPs can be subcultured to produce large amount of brown/brite adipocytes and cryopreserved. This model offers a platform for the identification of pathways governing the earliest steps of human adipocyte development as well as an abundant source of cells for cell therapy approaches.

Introduction

In mammals, three types of adipocytes coexist, i.e brown, beige and white adipocytes, which are all involved in energy balance regulation while having opposite functions. White adipose tissue \(WAT) is dispersed throughout the body and is mainly involved in energy storage and mobilization in form of triglycerides. In contrast, brown adipose tissue \(BAP) is specialized in energy expenditure. Brite, also named beige, adipocytes were recently described as brown-like adipocytes and represent a third type of adipocytes recruited in WAT[1]. BAT implants were recently shown to improve the metabolic conditions in obese mice and restore normoglycemia in diabetic mice[2, 3]. These recent findings offer promising prospects to counteract obesity by activating BAT or transplanting brown, beige adipocyte progenitors \(APs) in obese patients. However, BAT represents a minor fraction of adipose tissue in humans and disappears from most areas with age, persisting only around deeper organs[4]. Human BAT is hard to isolate in this regard, so an alternative cellular source is required to generate brown adipocytes. Human induced pluripotent stem cells \(hiPSCs) can be differentiated into multiple cell types in vitro. Following the pioneer work of Yamanaka's group on the generation of patent –specific hiPSC by reprogramming somatic cells[5], hiPSCs emerged as an unlimited source of autologous BAPs for cell-based therapy of obesity. Indeed, several studies indicated that hiPSCs could potentially be used to generate brown adipocytes with therapeutics properties. Nakao's group was the first to demonstrate the capacity of hiPSC to generate functional adipocytes[6]. Nishio et al.[7] developed a procedure to generate functional brown adipocytes at a high frequency using an hematopoietic cocktail to induce hiPSC differentiation. Interestingly, hiPSC-brown adipocytes were able to improve glucose tolerance after transplantation in mice. However, numerous issues have to be investigated before a therapeutic use of hiPSC-BAPs, notably their purification and differentiation into functional adipocytes. Indeed, in both works, total differentiated hiPSC cell populations, but not purified APs, were transplanted into mice. Differentiated hiPSC cultures can be enriched with adipocytes, but also contain other cell types that are unsuitable for transplantation, including undifferentiated hiPSC that can for teratomas. As well, Ahfeldt et al.[8] were able to generate pure brown APs from hiPSCs that displayed a high adipogenic capacity but only following transduction with adipogenic master genes. The need to genetically modify hiPSC-APs clearly illustrates the low adipogenic

potential of hiPSC-derived APs and hampers their clinical use. We recently reported the potential of hiPSCs to generate both brown and white Aps[9]. However, a bottleneck is the weak adipogenic potential of hiPSC-derived APs compared to adult adipose tissue-derived APs[10]. This feature has be observed by us and others using different approaches to derive mesenchymal stem cells from hESC and hiPSC. Therefore the differentiation of hiPSC-BAPs requires a protocol different than those used to differentiate APs derived from adult tissues. We describe a protocol to derive BAPs from hiPSC and to differentiate them at a high efficiency without genetic modification. A schematic diagram of the timing for derivation and differentiation of hiPSC-BAPs is shown in Figure 1.

Reagents

DMEM low glucose \(Lonza Cat. no. BE12-707F) DMEM/F12 \(In Vitrogen Cat. no 11330-057) EBM basal medium \(Lonza Cat. no. CC-3124) EGM \(Lonza Cat. no. CC-4133) FBS \(Gibco Cat. no. 10270) PBS \(Lonza Cat. no. BE17-516F) Sodium L-Ascorbate \(Sigma Cat. no. A4034) Hydrocortisone \(Sigma Cat. no. H0396) Human Epidermal growth factor \(EGF, Gibco Cat. no. PH0314) SB 431542 \(Sigma Cat. no. S4317) 3-isobutyl-1-methylxanthine \(IBMX, Sigma Cat. no. I7018) Insulin \(Gibco Cat. no. 12585-014) Triiodothyronine \(T3, Sigma Cat. no. T-6397) Rosiglitazone \(Cayman Chemical Cat. no. 71740) Dexamethasone \(Sigma Cat. no; D-4902) Penicillin/streptomycin \(Lonza Cat. no DE17-603E) Trypsin 2.5% \(Invitrogen Cat. no 15090-046) Gelatin \(Sigma Cat. no. G1393) L-glutamine \(Lonza Cat. no. BE17-605E) FGF2 \(Peprotech Cat. no. 100-18B) 2-mercaptoethanol \(Sigma Cat. no M6250) Non-essential amino acid \(Invitrogen Cat. no 11140-050) Knock-out Serum Replacement \(KSR, Invitrogen Cat. no 10828-028) **Medium setup** _The hiPSC growth medium_ is DMEM/F12 medium supplemented with \(final concentrations) KSR \(20%) + FGF2 \(10 ng/ml)+ L-glutamine \(2 mM) + Non-essential amino acid \(0.1 mM) + 2-mercaptoethanol \(0.1 mM) + Penicillin/Streptomycin \(1%) **Caution**: This hiPSC growth medium is for the maintenance of undifferentiated hiPSCs on feeder layer. The hiPSC growth medium may be different in the absence of feeders. _The adipose progenitor proliferation medium_ is DMEM low glucose supplemented with \(final concentrations) FBS \(10%) + FGF2 \(5 ng/mL) + L-glutamine \(2 mM) + penicillin/streptomycin \(1 %). _The hiPCS-BAP differentiation medium_ is EBM medium supplemented with \(final concentrations) FCS \(0.1%) + T3 \(0.2 nM) + Insulin \(1 µg/ml) + Rosiglitazone \(1 µM) + IBMX \(0.5 mM) + Dexamethasone \(0.25 µM) + SB 431542 \(5 µM) + ascorbic acid \(25.5 µg/ml) + hydrocortisone \(4 µg/ml) + EGF \(10 ng/ml). See table 1. **Sequences of primers to characterize BAPs by real time PCR:** _hDIO2_ : Fw GTCACTGGTCAGCGTGGTTTT ; Rev TTCTTCACATCCCCCAATCCT _hPAX3_ : Fw ACACCGTGCCGTCAGTGAGT ; Rev TCGCTTTCCTCTGCCTCCTT _hHOXC8_ : Fw GTCTCCCAGCCTCATGTTTC; Rev TCTGATACCGGCTGTAAGTTTGC _hHOXC9_ : Fw GCAGCAAGCACAAAGAGGA; Rev CGTCTGGTACTTGGTGTAGGG _hHOXA5_ : Fw CCCAGATCTACCCCTGGATG; Rev CAGGGTCTGGTAGCGCGTGT **Sequences of primers to characterize BAP-differentiated progenies:** _hUCP1_: Fw GTGTGCCCAACTGTGCAATG, Rev CCAGGATCCAAGTCGCAAGA _hPLIN1_: Fw ACCATCTCCACCCGCCTC; Rev GATGGGAACGCTGATGCTGT **Antibodies used to characterize BAP-differentiated progenies:** hUCP1 \(Calbiochem Cat. no. 662045) hPLIN1 \(Acris Antibodies Cat. no.

BP5015) **Antibodies used to characterize undifferentiated hiPSCs by FACS analysis:** Tra-1-60 \(Abcam Cat. No. ab16288) SSEA4 \(Abcam Cat. no. ab16287) **Antibodies used to characterize BAPs by FACS analysis:** CD73 \(Miltenyi Biotec Cat. no. 130-095-182) CD105 \(RD Systems Cat. no. FAB10971F) CD90 \(Miltenyi Biotec Cat. no 130-097-930) CD140a \(BD Pharmingen Cat. no. 556001) CD45 \(BD Pharmingen Cat. no. 555483) CD31 \(BD Pharmingen Cat. no. 555446) **Note**: ascorbic acid, hydrocortisone and EGF 1000 X are commercially available as EGM \(Lonza Cat. no. CC-4133).

Equipment

Tissue culture equipment Laminar flow tissue culture hood Incubator at 37°C with 5% CO2 Sterile 15-50 ml conical tubes 10 cm tissue-culture-grade plates \(Sarstedt cat. no. 83-3902) 24 well-tissue-culture-grade plates \(Sarstedt cat. no. 83-3922) 6 well-tissue-culture -grade plates \(Sarstedt cat. no. 83-3920) 6 well-plate-ultra-low-attachment surface plates \(Corning cat. no. 3471) 6 well-plates coated with gelatin: Prepare a solution of sterile 0.5 % gelatin in PBS. Warm the solution to 37°C. Then, add 1ml of gelatin per well of 6-well-tissue-culture-grade plates. Incubate 10 minutes at room temperature. Aspirate the gelatin before adding cells. Pasteur pipettes 3 ml. Sterile individually wrapped \(Sarstedt Cat. no. 86-1171001).

Procedure

Derivation of brown adipose progenitor \(BAP) from hiPSCs 1. Maintain undifferentiated hiPSC cells in hiPSC growth medium on 6-well-tissue-culture-grade. 2. Initiate the differentiation of hiPSCs by floating cultivation to form embryoid bodies \(EBs). As a guideline, seed hiPSCs from one well of 6-well-tissue-culture-grade plates onto one well of 6-well-ultra-low attachment plates in hiPSC growth medium without FGF2. This is taken as the day 0 of differentiation. **TIP**: Do not move the plate for 3 days in the incubator. This will improve the formation of EBs. 3. Change the hiPSC growth medium without FGF2 at day 3 and then change the medium daily to day 10. **TIP**: To change the medium, collect the medium containing EBs in a 15 ml conical tube. Leave the tube 15 minutes under the hood for allowing EBs to sediment at the bottom of the tube by gravity. A short spin of the tubes at a low speed \(10 sec at 800g) may increase the number of EBs collected. Remove gently the supernatant, add fresh medium and seed EBs back on the 6-well-ultra-low attachment plates. It is important to not dissociate EBs formed by using un-appropriate pipettes. We recommend the use of 3 ml sterile Pasteur pipettes. 4. At Day 10, plate EBs from one well of 6-well-ultra-low-attachment plate onto one gelatin-coated well of 6-well-plate-tissue-grade-culture in hiPSC growth medium. **TIP**: Do not move the plate for 3 days to improve adhesion of EBs on the bottom of the well. 5. Then, change the medium every day. 6. At day 17, wash EB outgrowths twice with PBS. Remove PBS and replace the hiPSC growth medium to the adipose progenitor proliferation medium. Change the medium every day for a week. 7. At day 24, passage the cells using trypsin. Split one well of 6-well plate in one 10 cm tissue culture grade plate. Maintain cells in adipose progenitor proliferation medium. This is determined as passage number 1. 8. Split cells with a 1:3 split ratio when reaching 80% of confluence. 9. Repeat step 8, usually 3-4 times, until achieving a cell population with a homogenous CD73 labelling determined by FACS analysis. Then, BAPs must be

characterized in more details as CD105+/CD90+/CD140a+/CD31-/CD45- by FACS analysis and by expression of _PAX3_, _DIO2_ and absence of expression of _HOXC8_, _HOXC9_, _HOXA5_ by real time PCR. **Note**: At that stage adipose progenitors can be amplified and cryopreserved using conventional methods \(as an example, see reference \[11]. _Differentiation of hiPSC-brown adipose progenitor_ 1. Plate BAPs at a high density, i.e., 1-5x10^4 cells/cm2 and maintain cells in the adipose progenitor proliferation medium. 2. When cells reach confluence, determined as the day 0 of differentiation, wash cells once with PBS and change the adipose progenitor proliferation medium to the hiPSC-BAP differentiation medium. 3. Three days after, change the hiPSC-BAP differentiation medium but with no IBMX and no dexamethasone. 4. Change the BAP differentiation medium with no IBMX and no dexamethasone once a week.

Timing

Derivation of homogenous BAP population from differentiating hiPSC takes 5-8 weeks and requires 4-5 passages. As a guideline, once BAPs have been derived to homogeneity, one 10 cm culture plate may contain 3x10^6 cells. The BAP Population Doubling time is around 30 hours. BAPs can be frozen at passage 5 and can be amplified to passage 15 with no loose of their adipogenic capacity. The first adipocytes filled with lipid droplets should appear 10 days after induction of the differentiation. The cultures can be maintained up to 35 days.

Troubleshooting

hiPSCs do not form EBs Check the undifferentiated immunophenotype of hiPSC cultures. 90%-100% cells of the culture should be Tra-1-60+/SSEA4+/CD73-/CD105-. The concentration of undifferentiated hiPSCs is too low. As a guideline, undifferentiated hiPSCs contained in one well of 6-well-tissue culture plates is seeded onto one well of 6-well-ultra-low attachment plates. Avoid disaggregating hiPSC colonies into single cells or into too tiny-clumps before seeding onto 6 well-ultra-low-attachment plates. _Differentiation of hiPSC-BAPs at a low efficiency_ Cell confluence is critical before inducing the differentiation. Check the different reagents added to the differentiation medium. It is recommended to aliquot each compound of the differentiation cocktail, to add them extemporaneously when preparing the differentiation. Avoid to re-freeze working aliquots.

Anticipated Results

Derivation of BAPs from hiPSCs Small cell aggregates should be visible at day 3 of EB formation. Single cells and cell debris can also be observed. These latter are eliminated during the first changes of medium. The size of EBs should increase till day 10. After day 10, EBs outgrowths expand and various cell types with different morphology should appear. Addition of adipose progenitor proliferation medium leads to a high cellular mortality. The percentage of CD73-positive cells increases at each passages and cells acquire a fibroblast-like morphology. _Differentiation of hiPSC-BAPs_ Adipocytes should appear 10-15 days after induction of the differentiation. The number of adpocytes increases with time of culture. A

high differentiation efficiency is obtained 20-30 days after induction as between 60-80% of hiPSC-BAPs undergo adipocyte differentiation.

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Figures

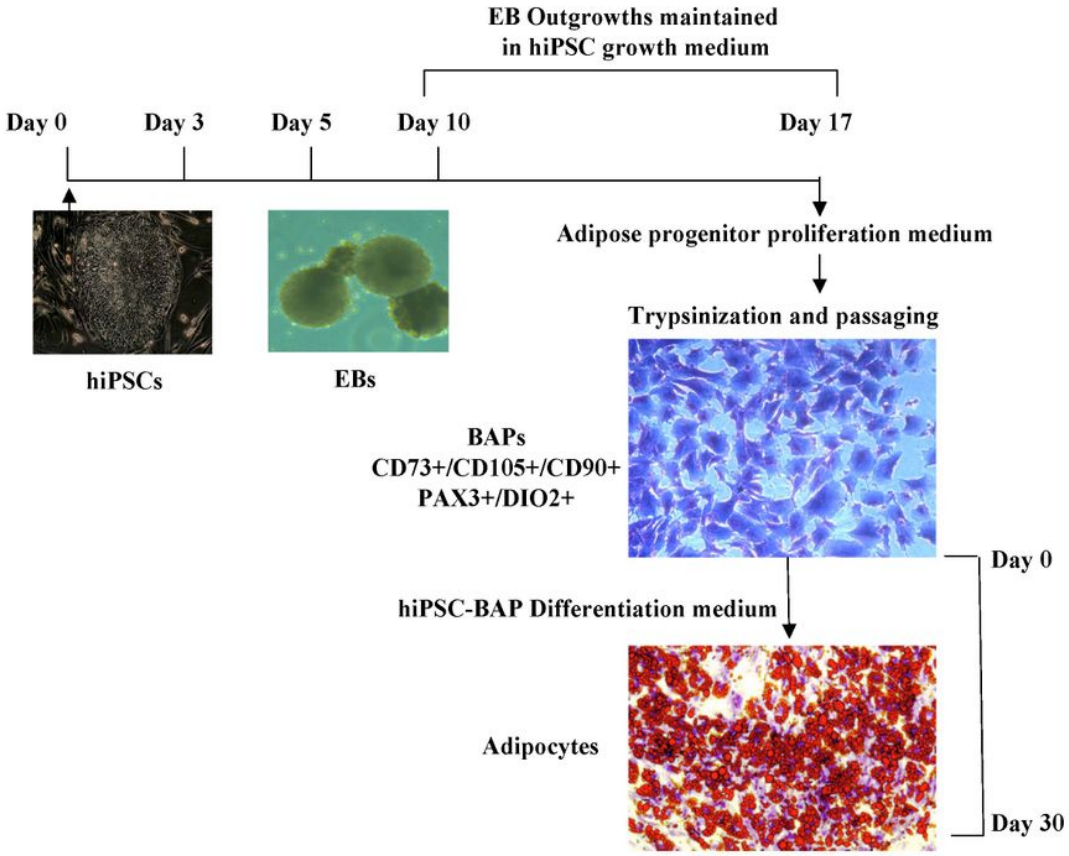

Figure 1

Schematic diagram for hiPSC-BAP derivation and differentiation Undifferentiated BAPs were stained with Cristal Violet and adipocyte progenies were stained with Oil Red O for lipid droplets.

hiPSC-BAP differentiation medium

Composition (concentration of stock reagent)	Volume for 100 ml	Final concentration
EBM	98 ml	
FBS	0.1 ml	0.1%
Dexamethasone (1 mM)	0.025 ml	0.25 μM
T3 (2 μM)	0.01 ml	0.2 nM
Insulin (4 mg/ml)	0.025 ml	1 μg/ml
IBMX (100 mM)	0.5 ml	0.5 mM
Rosiglitazone (10 mM)	0.01 ml	1 μM
SB 431542 (10 mM)	0.05 ml	5 μM
Ascorbic Acid (25.5 mg/ml)	0.1 ml or 0.1 ml of contained in CC4133	25.5 μg/ml
Hydrocortisone (10 mg/ml)	0.04 ml or 0.1 ml of contained in CC4133	4 μg/ml
EGF (10 μg/ml)	0.1 ml or 0.1 ml of contained in CC4133	10 ng/ml

Note: Dexamethasone and IBMX are omitted from the medium at day 3 of differentiation.

Figure 2

Table 1 hiPSC-BAP differentiation medium setup