Biochemical and Biophysical Research Communications 444 (2014) 543–548

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

The phenotype and tissue-specific nature of multipotent cells derived from human mature adipocytes

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ARTICLE INFO

Article history:
Received 14 January 2014
Available online 30 January 2014

Keywords:
Dedifferentiated fat cells
Adipocytes
Adipose-derived stem cells
Multipotent capacity
Dil-ac-LDL uptake

ABSTRACT

Dedifferentiated fat (DFAT) cells derived from mature adipocytes have been considered to be a homogeneous group of multipotent cells, which present to be an alternative source of adult stem cells for regenerative medicine. However, many aspects of the cellular nature about DFAT cells remained unclarified. This study aimed to elucidate the basic characteristics of DFAT cells underlying their functions and differentiation potentials. By modified ceiling culture technique, DFAT cells were converted from human mature adipocytes from the human buccal fat pads. Flow cytometry analysis revealed that those derived cells were a homogeneous population of $CD13^+$ $CD29^+$ $CD105^+$ $CD44^+$ $CD31^-$ $CD34^-$ $CD309^-$ α-SMA^- cells. DFAT cells in this study demonstrated tissue-specific differentiation properties with strong adipogenic but much weaker osteogenic capacity. Neither did they express endothelial markers under angiogenic induction.

1. Introduction

Human bone tissue and subcutaneous white adipose tissue are the most accessible sources of autologous adult stem cells or mesenchymal stem cells (MSCs). Considering the factors involving the cell number upon harvest, pain and morbidity to the donor [1], the adipose tissue presents to be a more favorable choice. Adipose-derived stem cells (ASCs) isolated from the stromal vascular fraction (SVF) were considered to be the key mesenchymal stem cells within this tissue, which are readily induced to differentiate toward adipocytes, osteocytes, chondrocytes, myocytes, neurons and etc. [2–4]. However, because the SVF of adipose tissue is highly complex in structure and cellular composition, the efficiency of ASCs harvest method so far also needs to be improved. Another obstacle for their application is that ASCs are constantly identified to be heterogeneous population composed of cells with various cellular characteristics and behaviors [4–7].

Mature adipocytes, another abundant cell group in fat tissue, have shown dynamic plasticity to be converted into fibroblast-like and lipid-free cells by a convenient method called ceiling culture technique [8,9]. Unlike the terminal differentiated adipocytes, this group of cells showed strong and steady proliferation ability and named dedifferentiated fat (DFAT) cells [8,10–12]. Compared with ASCs, DFAT cells are derived directly from adipocytes based on their unique buoyancy, which makes them a more homogeneous population. However, the cellular nature and differentiation stage of DFAT cells remained unclarified. Several groups reported that the DFAT cells demonstrated MSCs characteristics with the multipotent capacity of differentiating into adipocytes [9,13–15], osteoblasts [13,15,16], chondrocytes [13], skeletal myocytes [17], smooth muscle cell lineage [18,19], cardiomyocytes [20] and endothelial cells [21,22] in vitro. Additional studies also reported neural and neuronal progenitor markers were found their expression [23,24]. There were also other studies suggesting that the DFAT cells might be in a late stage of differentiation process and had more similarity with pre-adipocytes such as 3T3-L1 and 3T3-F442A cells, two novel pre-adipocyte cell lines isolated from Swiss 3T3 cells [25,26].

In this study, we isolate mature adipocytes from human buccal lipid pad and convert them into DFAT cells. The adipogenic capacity of DFAT cells were compared with the results of osteogenic induction. We also tested that their behavior under angiogenic environment. This study aims to analyze the multipotent characteristics of human DFAT cells and provide more evidence to clarify their cellular nature.

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http://dx.doi.org/10.1016/j.bbrc.2014.01.077
0006-291X/

2. Materials and methods

2.1. Cell culture and preparation

Human white adipose tissues were obtained from the buccal fat pad area of the patients receiving maxillofacial plastic surgeries in the West China Hospital of Stomatology, Sichuan University. The procedures were granted with the patients' full consent, as well as the approval from the Ethics Committee of State Key Laboratory of Oral Diseases, West China Hospital of Stomatology, Sichuan University. Without specification, all chemical and reagents in the following experiments were purchased from Sigma–Aldrich (St. Louis, USA).

DFAT cells culture utilized a modified method of the ceiling culture technique introduced previously by Sugihara [8]. Briefly, harvested adipose tissues were minced into small pieces in warm phosphate buffer solution (PBS) and digested in Dulbecco's modified Eagle's medium (DMEM, Invitrogen, Canada) with 0.1% Type II collagenase at 37 °C for 30 min. After filtration and repeated centrifugation, the floating adipocytes were collected and transferred into culture flasks (Falcon, Becton Dickinson Labware, USA) completely filled with high glucose DMEM containing 20% fetal bovine serum (FBS, J R Scientific, Inc., Canada) and kept inverted at 37 °C and 5% CO_2. After about 2 weeks that all adhered adipocytes lost their lipid drops, the flasks were re-inverted and the medium was changed every 4 days until the culture confluence was reached.

Purchased HUVECs (Lonza Walkersville Inc., USA) were maintained in EGM-2 medium (EBM-2 endothelial culture medium supplemented with EGM-2 MV kit, Lonza Walkersville Inc., USA). Without specification, 5th–8th passages of the DFAT cells and HUVECs were used in all experiments.

2.2. Flow cytometry

HUVECs and DFAT cells cultured in DMEM with 20% FBS as well as in EGM-2 medium were subjected to flow cytometry analysis. At 85–90% confluence, cells were detached by 0.25% trypsin–EDTA and suspended at a density of 1×10^6 cells/ml in PBS. Each sample was incubated with mouse anti-human antibodies including phycoerythrin conjugated CD13 (PE-CD13, aminopeptidase N), PE-CD29 (integrin b1), PE-CD31 (platelet endothelial cell adhesion molecule, PECAM), PE-CD34, fluorescein isothiocyanate conjugated CD44 (FITC-CD44, hyaluronate receptor, phagocytic glycoprotein-1), PE-CD105 (endoglin), PE-CD106 (vascular cell adhesion molecule-1, VCAM-1), PerCP-CD309 (vascular endothelial growth factor receptor 2, VEGFR-2), and PE-α-SMA (alpha-smooth muscle actins), respectively. Corresponding PE conjugated mouse IgG antibodies were used as isotype control. Anti-α-SMA antibody was obtained from R&D Systems (USA), while others were purchased from BD Biosciences (USA). Flow cytometry recording and analysis were performed with the Guava EasyCyte Mini System and the CytoSoft Software (Guava Technologies, USA).

2.3. Differentiation analysis

DFAT cells were planted onto 35 mm culture dishes (Falcon, Becton Dickinson Labware, USA) at a density of 5×10^4 cells/dish with induction medium for 1, 2, 3 and 4 weeks, respectively. Adipogenic differentiation induction medium was high glucose DMEM containing 20% FBS, 0.5 mM 3-isobutyl-1-methylxanthine, 1 μM dexamethasone and 5 μg/ml insulin-transferrin-selenium-X. Osteogenic differentiation induction was high glucose DMEM containing 10% FBS, 10 nM dexamethasone, 10 mM L-Ascorbic Acid and 50 μM β-Glycerophosphate. At each indicated time, cells were fixed with 4% paraformaldehyde (Wako Pure Chemical Industries, Japan) for 30 min. Lipid accumulation was examined by staining with 3 mg/ml Oil Red O for 30 min at room temperature. Calcium deposit was visualized by 20 mg/ml Alizarin Red S staining for 5 min at room temperature.

2.4. Angiogenic induction and analysis

At 50% confluence in 35 mm dishes, the culture medium of DFAT cells was replaced by EGM-2 medium containing vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF) and epidemic growth factor (EGF). DFAT cells and HUVECs of the same passage were also cultured in DMEM with 20% FBS and EGM-2, respectively. At indicated time, cells were fixed by 2% formaldehyde under 4 °C for 20 min and incorporated with rabbit anti human von Willebrand factor (vWF) as the primary antibody, and Alexa-568 goat anti rabbit immunogloblulin (Molecular probes, Invitrogen, USA) as the secondary antibody. Nuclei were counterstained with 4′,6′-diamidino-2-phenylindole (DAPI). All incubations above were performed under 4 °C with proper light protection. Flow cytometry analysis was also applied with antibodies against CD31, CD309 and α-SMA.

2.5. Dil-ac-LDL uptake analysis

DFAT cells and HUVECs were plated onto the 35 mm dishes at 5×10^4 cells/dish. At 75% confluence, the culture medium was replaced by the serum-free DMEM for 24 h, followed by incubation with 2 μg/ml 1,1′-dioetadeeyl-3,3,3′,3′-tetramethylindocarboeyanine labeled acetylated low density lipoprotein (Dil-ac-LDL) for 5 h at 37 °C and 5% CO_2. Then cells were washed and fixed with 4% paraformaldehyde under 4 °C for 30 min followed by DAPI staining for 3 min. The Dil-ac-LDL uptake was assessed with LSM710 microscopy.

2.6. Statistical analysis

Data were expressed as mean ± SD. Statistical analysis was performed with two-tail Student's t test for double-group comparisons or one-way ANOVA for multiple comparisons under SPSS 13.0. $P < 0.05$ was considered to be significant.

3. Results

3.1. Establishment of human DFAT cells from mature adipocytes from the buccal adipose tissue and their characterization

The purity of isolated adipocytes prior to ceiling culture is crucial to avoid contamination of DFAT cells with other cellular lineages [21,25]. After 4–5 days of culture, the single lipid drop within the plasma began to break into dozens of small droplets, while no outgrowth of other cell was observed (Fig. 1, A). At the 12th–14th day, with all lipid portions disappeared, the dedifferentiated cells changed into fibroblast-like shape and regained vigorous proliferation ability (Fig. 1, B–D). In this study, the dedifferentiation process and outcome of human adipocytes derived from buccal fat pads resembled the findings in the previous reports using abdominal subcutaneous adipose tissues [9,20,25,26]. There was no obvious decrease of proliferation ability or spontaneous differentiation observed up to the 25th passage.

3.2. Phenotype characteristics of DFAT cells

Flow cytometry analysis showed that human DFAT cells obtained in this study are a homogeneous population with uniformly expression of all selected markers, including positive for CD13, CD29, CD105, and CD44, but negative for CD31, CD34, CD309,

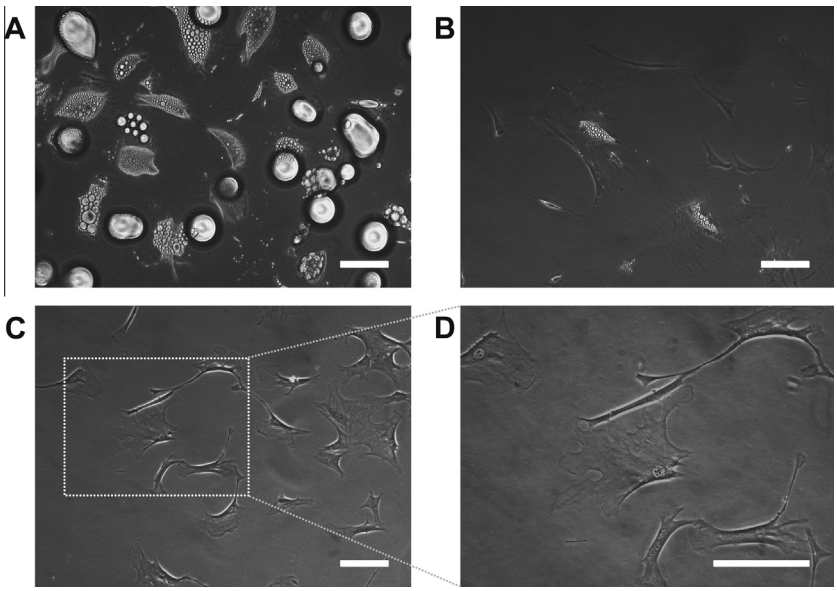

Fig. 1. Establishment of the human DFAT cells. (A) At the 4th–5th day, most of the adhered adipocytes lost their unilocular outlook and presented multiple droplets. (B) Content of the lipid portion continued to decrease significantly and gradually disappeared after around two weeks of ceiling culture. (C, D) DFAT cells exhibited various sizes with big flat cell bodies, single nucleus and multiple processes. The size of DFAT cells might be determined by their ancestor adipocytes based on the different amount of intracellular lipid portion. (Bar length: 100 μm).

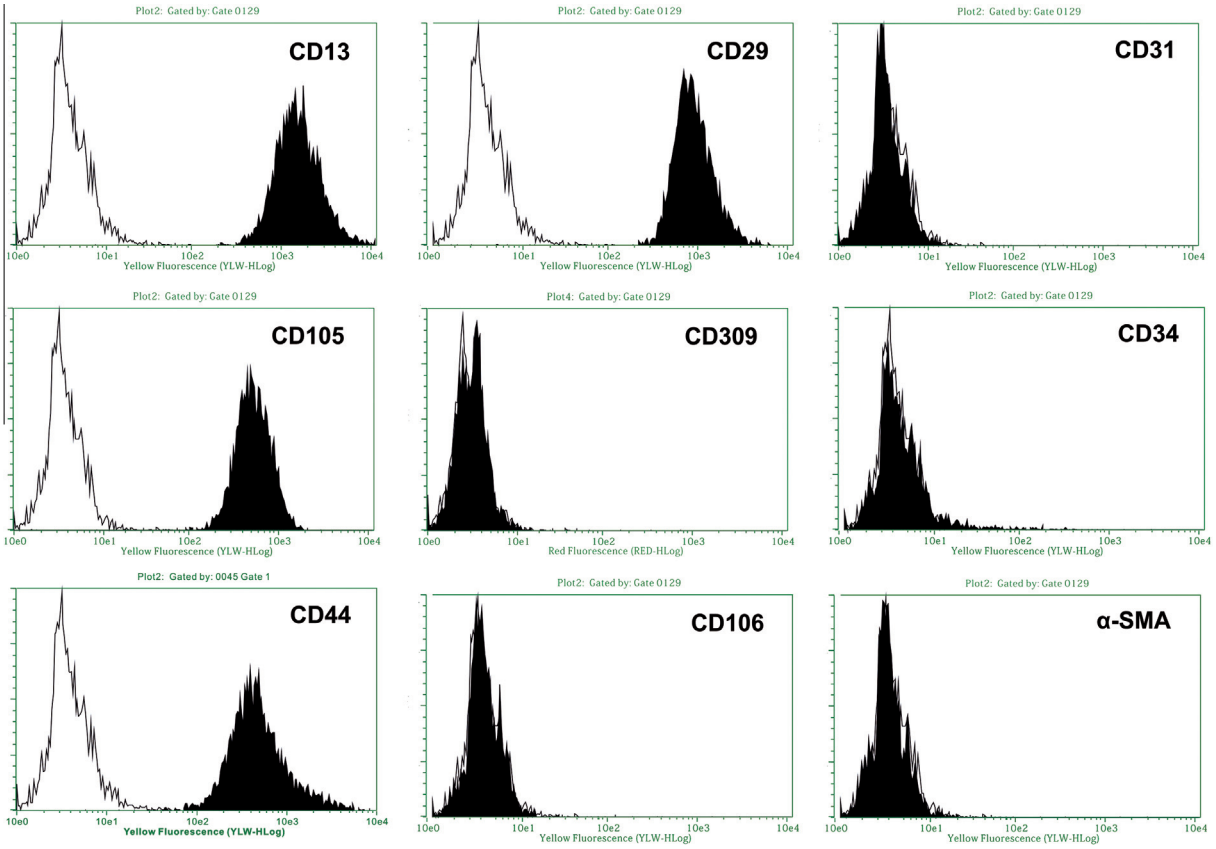

Fig. 2. Phenotype profiling of human DFAT cells by flow cytometry. All markers selected for the flow cytometry analysis demonstrated uniform expression. The human DFAT cells in this study could be characterized as a CD13 (99.78 ± 0.02%), CD29 (99.50 ± 0.23%), CD105 (98.12 ± 1.66%) and CD44 (99.46 ± 0.31%) positive, but CD31 (3.24 ± 0.45%), CD34 (9.62 ± 0.89%), CD309 (2.66 ± 0.33%), CD106 (4.48 ± 0.62%) and α-SMA (4.66 ± 0.46%) negative cell population. Data were obtained by averaging 6 samples for each marker.

CD106 or α-SMA compared with the isotype control (Fig. 2). Such expression pattern was also considerably stable and persistent from the 1st passage to as far as the 25th passage. The phenotype profile above was consistent with previous report [13].

3.3. The adipogenic and osteogenic differentiation assay of DFAT cells

Under the adipogenic induction, DFAT cells reestablished fat drops within their plasma at 5th–7th day (Fig. 3). The adipose droplets continued to increase and merge into larger ones. After 3 weeks of induction most of the cells exhibited positive for Oil Red O. This result was consistent with previous observations in vitro and in vivo that DFAT cells could redifferentiate into adipocytes [13,25,26]. However, using the similar induction recipe with other studies containing dexamethasone and β-glycerophosphate [26], osteogenic differentiation ability of DFAT cells in our experiment was significantly weaker. After 3 weeks of osteogenic culture, the DFAT cells demonstrated limited mineralized matrix indicated by Alizarin Red S staining, while a relatively large portion of cells turned into multilocular appearance and positive adipose staining (Fig. 3). Adjusting the amount of dexamethasone and β-glycerophosphate in the culture medium or extending the induction time to 5 weeks didn't brought obvious change to this result (data not shown). Although, the expression of osteogenic transcription factors in DFAT cells such as Runx2, osteopontin, osterix and osteocalcin were confirmed in previous reports [5,9,13], DFAT cells in this study exhibited lower potential of differentiating toward osteoblasts than adipocytes in vitro.

3.4. Angiogenic induction of human DFAT cells

Compared with the control groups, inducted cells showed dramatic changes that the original flat cell bodies quickly shrunk with several processes extended (Fig. 4, A). After 5 days of culture, almost all cells become thin spindle-shaped with long bipolar-like processes and assembled in definite directions (Fig. 4, A). Immunofluorescent staining demonstrated that, comparing with HUVECs, endothelial cell specific marker vWF was not found in DFAT cells before or after induction (Fig. 4, B). Results of flow cytometry analysis were also consistent with the findings above. Common endothelial cell markers such as CD31, CD34 and CD309, which were expressed by HUVECs, were all negative on DFAT cells before and after induction (Fig. 4, C).

3.5. Dil-ac-LDL uptake test of DFAT cells under different culture conditions

Dil-ac-LDL is generally considered to be taken up by macrophages and endothelial cells via scavenger cell pathway of LDL metabolism. However, uptake of Dil-ac-LDL was found on rat brain microvascular pericytes, with a lower incorporation ratio compared with brain endothelial cell lines [27]. In this study compared with HUVECs, DFAT cells demonstrated significant incorporation ability of Dil-ac-LDL that all cells were positively stained even after cultured with a relative low density of LDL (Fig. 4). Changed into endothelial cell culture environment, this ability didn't have obvious change (Fig. 4). Further experiments with cells of the 20th and 25th passages also produce the same results. Internalization of LDL by DFAT cells indicated that the presence of lipoprotein receptors on their plasma membrane. This finding could be perceived as that during the dedifferentiation process of mature adipocytes, the original lipoprotein receptors probably remained.

4. Discussion

In this study, by modified ceiling culture technique, human DFAT cells were derived from the mature adipocytes from human buccal fat tissues. Ceiling culture has been reported to be a reliable

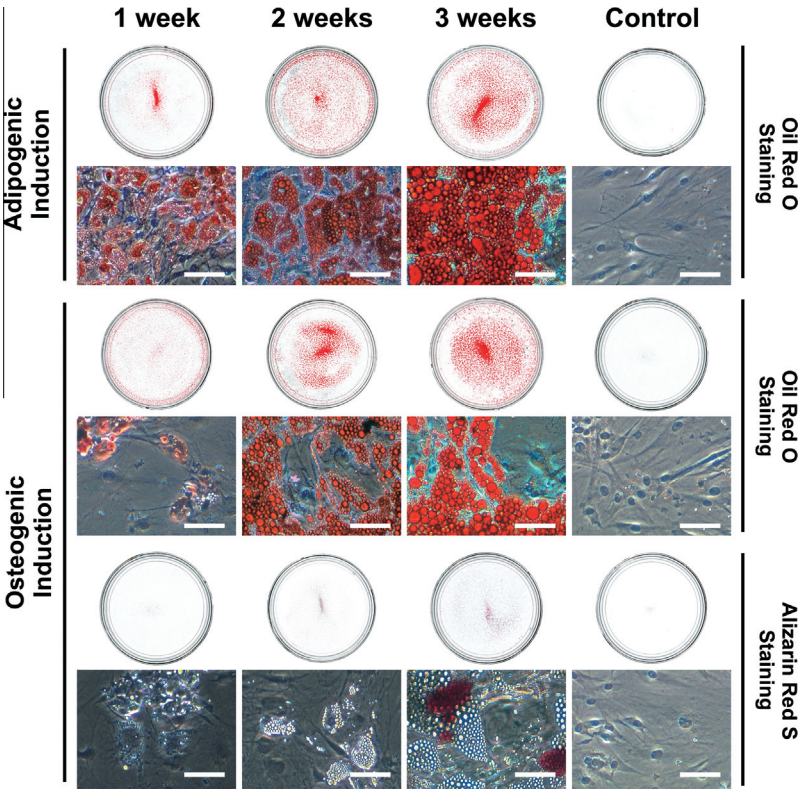

Fig. 3. Adipogenic and osteogenic differentiation of human DFAT cells (bar length: 50 μm). For adipogenic induction, lipid drops were first observed after 5–7 days and accumulated quickly in the following weeks indicated by positive staining of Oil Red O. For osteogenesis, positive Alizarin Red S staining was found after 3 weeks. However, adipose staining was also confirmed at the 1st week. At the end, differentiation into osteoblasts was relatively limited and accompanied with spontaneous redifferentiation into adipocytes.

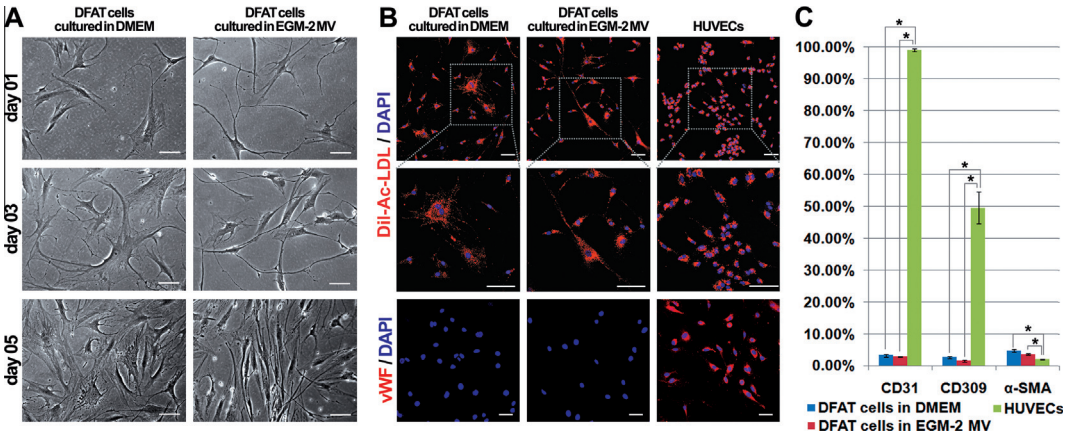

Fig. 4. Angiogenic induction of human DFAT cells. (A) In the endothelial culture medium, morphology changes of DFAT cells were significant and quick (bar length: 50 μm). (B) The upper: Dil-ac-LDL internalization of DFAT cells and HUVECs at the 7th day of culture (bar length: 50 μm).The lower: Immunofluorescent staining and confocal microscopy evaluation after 7 days of angiogenic induction (bar length: 50 μm). DFAT cells cultured in endothelial medium were negative for vWF. (C) Flow cytometry analysis. Before and after 7 days of angiogenic culture, DFAT cells in this study kept an constant negative expression for endothelial cell markers CD31 (3.24 ± 0.45% and 2.84 ± 0.17%, respectively) and CD309 (2.66 ± 0.33% and 1.56 ± 0.29%, respectively), compared with HUVECs. *: $P < 0.05$.

method to harvest a highly homogeneous fraction of mature adipocytes at a percentage over 99% [25,26]. The dedifferentiation process of the mature adipocytes, as well as the morphology and proliferation ability of the obtained DFAT cells (Fig. 1), was consistent with previous reports [9,13,20,25,26]. The purification of the cell culture was further confirmed by the uniform expression for each selected marker in flow cytometry analysis (Fig. 2). Dedifferentiation of adipocytes could be induced not only in experimental culture condition, but also take place in certain pathological situations of liposarcoma [28]. Similar phenomenon also be found on other mesenchymal cell lineages including chondrocytes [29] and muscle cells [30], when these cells were also subjected to ischemic or hypoxia conditions. Similarly, low-oxygen ischemic-like environment was considered to trigger the loss of the lipid substance and consequently lead to the dedifferentiation changes although the underlying molecular mechanism remained unclear [9].

The cellular nature of DFAT cells has not been clarified. Several studies have already claimed that they were a group of MSCs with similar characteristics of ASCs, according to the marker profiles and multipotent differentiation assay [13,15]. But the same research team also classified them into pre-adipocytes because of the genetic comparison with other novel pre-adipocytes [25,26]. Our differentiation test suggested that DFAT cells in this study shared more characteristics with pre-adipocytes, not completely in conformity with the identified vascular wall resident endothelial progenitor cells, subendothelial progenitor cells, vascular wall hematopoietic progenitor cells and white fat progenitor cells, based on the former report [31].

Previous studies [25,26] established pre-adipocyte cell line from mature adipocytes, which could proliferate continuously and had the ability to redifferentiate into adipocytes spontaneously [32]. While DFAT cells derived from dedifferentiated mature adipocytes using the ceiling culture method in the absence of any specific factors [25,26,32], which could redifferentiate into adipocytes under proper induction[9,13–15]. Both had the histological origin in common and DFAT cells had experienced a unique pre-adipocyte cell line before the terminal differentiation into mature adipocyte [33]. In this study, DFAT cells exhibited more active adipocyte differentiation inclination under the condition of osteogenic differentiation induction medium and Dil-ac-LDL uptake analysis showed the original lipoprotein receptors probably remained during the dedifferentiation process of mature adipocytes. These evidence including the negative conclusion of its differentiation toward endothelial cells could prove that DFAT cells remained certain mature adipocyte characteristics and hadn't lost the adipose-tissue-specific nature.

It was reported that DFAT cells could not only redifferentiate into lipid-filled adipocytes as same as pre-adipocyte, but also can trans-diffenentiate into other cell types under the proper conditions, including osteoblasts [13,15,16], chondrocytes [13], and myocytes [17–20] in vitro. Other studies in vivo also suggested that DFAT cells could regenerate fat pads, ectopic osteoid tissue or muscle tissue [13,15,18,20,25,26], and contribute to sphincter function [19], infarcted cardiac tissue repair [20] and central nervous system recovery [24,34]. However, in this study, DFAT cells showed much weaker osteogenic differentiation ability, and no trans-differentiation ability toward endothelial cells. The multipotent capacity of DFAT cells might be limited and conditional based on their tissue-specific. In addition, our experiment reveal that human DFAT cells did not expressed key endothelial cell markers such as CD31, CD34, CD309 or vWF (Fig. 2, 4) before or after the induction with common angiogenic cytokines. These findings were consistent with most previous reports on DFAT cells [13,22,23]. However, it contradicted with Medet Jumabay's research, in which the primary DFAT cells could undertake endothelial differentiation in vivo [22]. A possible explanation is that culture conditions might change the potency of pluripotent stem cells or reprogram adult stem/progenitor cells to endow them with a broader range of differentiation potential [35].

Acknowledgments

We would like to thank Dr. Atsunori Sugawara and Dr. Hiroyasu Kazuhiko for the technical assistance. This study is supported by National Natural Science Foundation of China (Grant Nos. 81000456 and 81170941).

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