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ORIGINAL ARTICLE

Comparative analysis of human UCB and adipose tissue derived mesenchymal stem cells for their differentiation potential into brown and white adipocytes

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Abstract

The differentiation potential of umbilical cord blood-derived mesenchymal stem cells (UCB-MSCs) into brown and white adipocytes in comparison to Adipose tissue derived MSCs (AD-MSCs) were investigated in order to characterize their potency for future cell therapies. MSCs were isolated from ten UCB samples and six liposuction materials. MSCs were differentiated into white and brown adipocytes after characterization by flow cytometry. Differentiated adipocytes were stained with Oil Red O and hematoxylin/eosin. The UCP1 protein levels in brown adipocytes were investigated by immunofluoresence and western blot analysis. Cells that expressed mesenchymal stem cells markers (CD34−, CD45−, CD90+ and CD105+) were successfully isolated from UCB and adipose tissue. Oil Red O staining demonstrated that white and brown adipocytes obtained from AD-MSCs showed 85 and 61% of red pixels, while it was 3 and 1.9%, respectively for white and brown adipocytes obtained from UCB-MSCs. Fluorescence microscopy analysis showed strong uncoupling protein 1 (UCP1) signaling in brown adipocytes, especially which were obtained from AD-MSCs. Quantification of UCP1 protein amount showed 4- and 10.64-fold increase in UCP1 contents of brown adipocytes derived from UCB-MSCs and AD-MSCs, respectively in comparison to undifferentiated MSCs ($P < 0.004$). UCB-MSCs showed only a little differentiation tendency into adipocytes means it is not an appropriate stem cell type to be differentiated into these cell types. In contrast, high differentiation efficiency of AD-MSCs into brown and white adipocytes make it appropriate stem cell type to use in future regenerative medicine of soft tissue disorders or fighting with obesity and its related disorders.

Keywords Umbilical cord blood-derived mesenchymal stem cells · Adipose tissue derived mesenchymal stem cells · Mesenchymal stem cells · Brown adipocyte · White adipocyte · Uncoupling protein 1 (UCP1)

Introduction

Two types of adipose tissue (AT) with different functions found in mammals, namely white adipose tissue (WAT) and brown adipose tissue (BAT). WAT store energy in the form of triacylglycerols (TAGs), but BAT turns the energy of TAGs to heat [1]. While WAT transplantation has been used as a material for plastic and reconstructive surgery to amend soft tissue damages caused by extensive burns, tumor removal surgery, or congenital defects [2], anti-obesity and antidiabetic effects of BAT in rodent models [3] make it an investigating material to find preventive and therapeutic approaches for obesity and obesity related complications including cardiovascular diseases, type-2 diabetes, and cancer [4].

Based on the literature, both adipose tissue and UCB are sources of MSCs. But, isolation of MSCs from UCB, obtained after full-term delivery of the newborn, in comparison to that from BM and AT, is completely safe, non-invasive, painless, and harmless for the mother and infant [5]. On the other hand, the immunogenicity of UCB-MSCs are less than the other stem cell sources, so UCB-MSCs

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seem safer for allogenic and autologous transplantations [6]. Because of these reasons, human UCB-MSCs are a good candidate to be used as an alternative for MSC transplantation. The effectiveness of UCB-derived hematopoietic stem cells (HSCs) for treating different hematological diseases has been established [7–9]. But there are contradictory reports about the presence and differentiation potential of UCB-MSCs [10–13].

To date, the differentiation potential of AD-MSCs [14, 15], BM-MSCs [16], and human pluripotent stem cells (PSCs) such as human embryonic stem cells (hESCs) and human inducible PSCs (hiPSCs) [17, 18] into brown adipocytes were described previously. However, there isn't any report about differentiation potential of UCB-MSCs into brown adipocytes. To fill this gap, in this study we compared isolation and in vitro differentiation potential of MSCs derived from UCB and AT, into both brown and white adipocytes.

Materials and methods

Ethical approval

Before the study was began, all procedures were approved by the Clinical Research Ethics Committee of Ege University Faculty of Medicine (Ethical Approval Number: 15-14.1/15). Informed consent was obtained from all individual participants included in the study.

In the present study, six abdominal liposuction materials collected from healthy adult females (BMI ≥ 30) undergoing plastic surgery in Department of Plastic and Reconstructive Surgery, Ege University Medical School Hospital, Izmir, Turkey.

Ten UCB samples were collected from healthy pregnant donors with single gestation pregnancy, after full-term delivery, in Department of Obstetrics and Gynecology of Ege University Medical School Hospital, Izmir, Turkey.

Isolation of UCB-MSCs and AD-MSCs

For UCB-MSCs isolation

50 ml UCB samples were collected using 50 ml syringes containing 5 ml citrate phosphate dextrose adenine-one (CPDA-I) from umbilical cord after full-term delivery. The samples were processed immediately to isolate MSCs. UCB was placed on Ficoll®-Paque PREMIUM 1.084 (GE Healthcare, 17-5446-02) with the same amount and mononuclear cells (MNCs) fraction was obtained by density gradient centrifugation at 600×*g* for 45 min at RT. After washing with PBS, MNCs were plated in 25 cm^2 culture flasks at a density of 2×10^6 cells per cm^2 containing Iscove Modified Dulbecco medium (IMDM) (Gibco, 31980030) supplemented with 20% FBS, 100 U/ml penicillin and 100 µg/ml streptomycin, 2 mmol/l L-glutamine, and 10 ng/ml basic fibroblast growth factor (bFGF). After 5 days of incubation at a 37 °C humidified atmosphere containing 5% CO_2, non-adherent cells were removed. Replacement of culture medium was done every 3 days until cells reached to 80% confluence.

For AD-MSCs isolation

MSCs were isolated from 300 ml liposuction materials using enzymatic digestion [19]. Briefly, lipoaspirates were washed with Dulbecco's phosphate buffered saline (DPBS) until the adipose layer becomes light yellow color. AT was treated with equal amount of collagen type IV (1%) (Sigma) and incubated in 37 °C for 30 min while swirling every 5–10 min intervals until adipose layer was completely digested. Enzyme activity was stopped by adding an equal volume of DMEM containing 10% FBS. Stromal vascular fraction (SVF) bellow adipose cell layer was collected and centrifuged at 1000×*g* for 5 min. SVF pellet was washed with DPBS and plated on 25 cm^2 culture flasks at a density of 2×10^4 cells per cm^2 containing DMEM-F12 (Gibco, 31331-028) supplemented with 20% FBS, 100 U/ml penicillin and 100 µg/ml streptomycin, 2 mmol/l L-glutamine, 10 ng/ml bFGF, and 5 ng/ml EGF. After 3 days of incubation in a humidified atmosphere containing 5% CO_2 at 37 °C, non-adherent cells were removed. Replacement of culture medium was done every 3 days until cells reached to 80% confluence.

Flow cytometry analysis

Flow cytometry analysis was performed to characterize the isolated MSCs based on cell surface markers. Cells in the third passage were harvested by 0.25% trypsin/EDTA and washed twice with DPBS. 5×10^5 cells were re-suspended in blocking buffer (1XPBS containing 2% BSA, 0.03% Sodium Acid, 5% FBS) and incubated in + 4 °C for 30 min. Thereafter, cell suspension was centrifuged at 1000×*g* for 5 min and incubated with fluorescein isothiocyanate (FITC)-conjugated mouse monoclonal antibodies against human CD34, CD45, CD90, and CD105 cell surface markers (ThermoFisher scientific) separately, in + 4 °C for 45 min. Cells washed and re-suspended in 100 µl 1× PBS for cell counting by BD Accuri C6 Flow Cytometer (BD Biosciences) and were analyzed by CFlow Sampler accuri software (BD Biosciences). Mouse isotype IgG antibodies were used as controls (ThermoFisher scientific).

Differentiation of MSCs into brown and white adipocytes

Brown adipocyte differentiation

MSCs were cultured on glass coverslips in 6-well plates until reaching to 80% confluence and incubated at 37 °C for approximately 24 h in DMEM-high glucose (Gibco Invitrogen) supplemented with 20% FBS and 100 ng/ml Bone Morphogenic protein7 (BMP7) (Thermofisher Scientific, PHC9541). Then, cells were cultured for 6 days in induction medium consisting of 0.2 µM 3,3′,5-triiodo-L-thyronine sodium salt (T3, Sigma, T6397), 1 µM dexamethasone (Sigma, D4902), 10 µg/ml apo-transferrin bovine (Sigma, T1428), 1 µM Insulin (Sigma, I0516), 500 µM 3-isobutyl-L-methylxanthine (IBMX, Sigma I7018), 0.2 mM indomethacin (Sigma, I7378) and 100 ng/ml BMP7 in DMEM-high glucose (ThermoFisher Scientific, 41965) with 20% FBS. Induction medium was changed with differentiation medium consisting of 0.2 µM T3, 10 µg/ml apo-transferrin, 1 µM insulin, 1 µM rosiglitazone (Sigma, R2408) and 100 ng/ml BMP7 in DMEM-high glucose with 20% FBS and cells were incubated at 37 °C for 3 weeks. All culture mediums were changed with 3 days intervals.

White adipocyte differentiation

One day after 80% confluence, MSCs were cultured in induction medium consisting of 0.2 µM T3, 1 µM Dexamethasone, 10 µg/ml apo-transferrin, 1 µM Insulin, 500 µM IBMX, and 0.2 mM indomethacin in DMEM-high glucose with 20% FBS. After 6 days, differentiation medium consisting of 0.2 µM T3, 10 µg/ml apo-transferrin, 1 µM insulin, and 1% filtered-sterile olive oil in DMEM-high glucose with 20% FBS was added and cells were incubated at 37 °C for 3 weeks.

Undifferentiated control MSCs were kept in its normal medium with 20% FBS.

Oil Red O staining

Visualization of lipid-rich vacuoles was carried out by Oil Red O staining. Cells washed with PBS and fixed by adding 4% paraformaldehyde (PFA) for 1 h in room temperature (RT). After washing with PBS, the cells were stained with a 0.2% Oil Red O solution for 15 min at RT. Nuclear staining was done using hematoxylin (Dako, Denmark, CS700). For quantification of lipid amount, differentiated cells were stained with Oil Red O and then were analyzed by ImageJ (https://imagej.nih.gov/ij/). Twenty fields in three replicates of each sample were counted.

Hematoxylin–eosin (HE) staining

The morphology of the differentiated adipocytes was investigated by HE staining. Cells washed with PBS and fixed by 4% PFA for 1 h in RT. After that more fixation was done by immersing cells in 100% ethanol for 30 s. Nuclear staining was performed by hematoxylin (Dako, Denmark, CS700) for 2.5 min. After washing with tap water for 5 min cytoplasm staining was done by Eosin (Dako, Denmark, CS701) for 2.5 min. Dehydrating was done with increasing concentrations of ethanol. After clearing with xylene slides were mounted using Histomount Mounting Solution (Invitrogen, 00-8030).

Immunofluorescence analysis of UCP1 protein in brown adipocytes

Cells washed with PBS and fixed by 4% PFA for 15 min. Blocking was performed using 2% BSA in PBS for 1 h at RT. Following fixation and blocking, cells were again incubated with 1:500 diluted mouse monoclonal anti-UCP1 antibody (Abcam, ab57687) for 1 h at RT. They were washed three times with PBS containing 2% Triton X-100 and then incubated with 1/200 diluted preadsorbed goat anti mouse IgG (Alexa Fluor® 488) (Abcam, ab150117) for 1 h at RT. After washing steps, cells were checked with fluorescent microscopy (Olympus, Tokyo, Japan) following the addition of 0.5 µg/ml of 4′,6-diamidino-2-phenylindole (DAPI).

Detection of UCP1 protein amount using western blotting analysis

A total of 1×10^6 cells of differentiated brown, white, and undifferentiated cells were washed twice with ice-cold PBS. Cell lysates were achieved by adding 100 µl of RIPA buffer (Thermo Scientific, 89900) containing complete, Mini, EDTA-free protease inhibitor cocktail (Roche Applied Science, 04693159001). The protein concentration of each sample was determined using the Pierce BCA Protein Assay Kit (Thermo Scientific, 23227) in accordance with the manufacturer's protocols. Equal amounts of protein samples (25 µg) were added to each well of 12% bis-tris sodium dodecyl sulfate–polyacrylamide gel electrophoresis (Novex, Invitrogen, Waltham, MA, USA) gel and protein separation was done at 200 V for 1 h. Separated proteins on the gel were transferred onto polyvinylidene difluoride membrane (Invitrogen) using iBlot Gel Transfer System (Invitrogen). The blocked and washed membrane was then exposed to 1:300 diluted mouse monoclonal anti-UCP1 antibody (Abcam, ab57687) and 1:2000 diluted anti-β tubulin monoclonal antibody BT7R (Thermoscientific, MA516308) and then incubated for 1 h at RT. Anti-mouse Western Breez Chromogenic Immunodetection kit (Novex, Invitrogen, WB7103) containing ready-to

use Alk-Phos-conjugated anti-mouse secondary antibody was used for chromogenic detection of the UCP1 and β-tubulin proteins according to the manufacturer's instructions. β-Tubulin was used to estimate the loading ratios of total protein. Quantification of Western blot results was done using ImageJ program (https://imagej.nih.gov/ij/).

Statistical analysis

The quantitative analysis of western blotting results and Oil Red O staining was evaluated via Student's *t* test using the SPSS 15.0 software (Chicago, IL, USA). Standard error of mean (SEM) was calculated with same software. P value < 0.05 was considered as significant. All experiments were repeated at least three times.

Results

MSCs isolation

Approximately 2×10^8 mononuclear cells were isolated from 50 ml UCB by Ficoll gradient centrifugation method. The adherent formless cells (Supplementary Fig. 1) appeared after 2 days of incubation in a 37 °C 5% CO_2 cell culture incubator. The medium was renewed 5 days after inoculation of mononuclear cells. The first cell colonies (Fig. 1a) and fibroblastic cells (Fig. 1b) appeared at the end of the first and second weeks, respectively. In average five cell colonies were achieved from 2×10^8 mononuclear cells. Cells were passaged after the third week (Fig. 1c). MSCs were isolated from seven samples out of ten UCB samples.

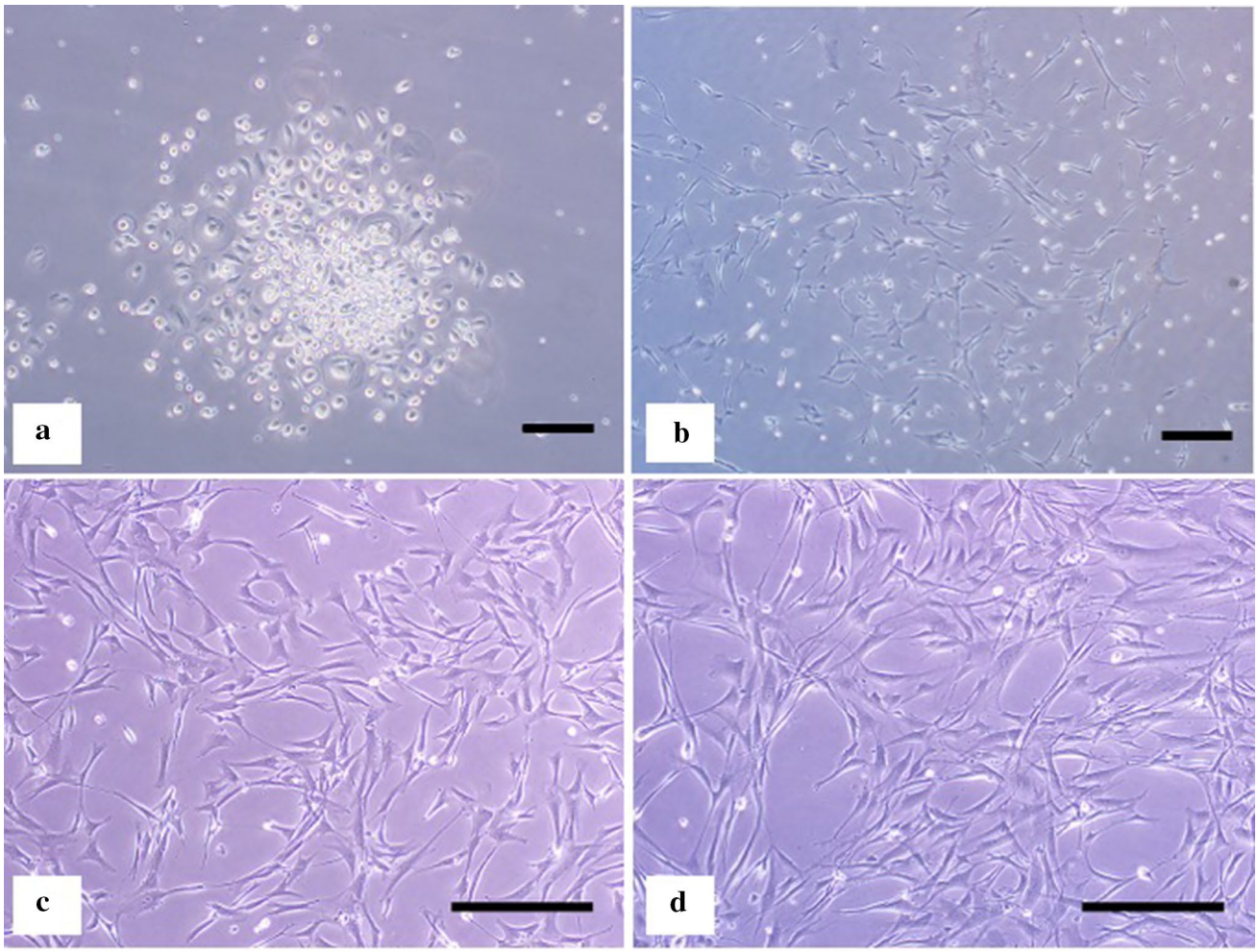

Fig. 1 Isolated UCB- and AD-MSCs (all scale bar = 200 μM). **a** The UCB-MSCs colonies on the 7th day. The Fusiform cells can be seen around cell colonies (objective 10×). **b** The fibroblastic cells appeared in the place of colonies at the end of the second week (objective 10×). **c** The image of UCB-MSCs at the third week (objective 20×). **d** AD-MSCs on 7th day (objective 20×). The stem cell colonies similar to that seen in UCB-MSCs did not appear in the cultured flasks of AD-MSCs. The AD-MSCs adhered to the plastic surface of cell culture flasks uniformly

Approximately 1×10^9 cells were isolated from 300 ml lipoaspirate material. These cells were cultured in five 75 cm^2 flasks. After 72 h incubation at 37 °C nonadherent cells were removed. As shown in Fig. 1d, the cells were passaged when covered > 80% of cell culture flask surface on the 7th day. MSCs were isolated from all lipoaspirate samples (100%).

Flow cytometry results

In this study, MSC surface markers including CD90 and CD105, and hematopoietic surface markers, CD34 and CD45, were used to characterize isolated cells from UCB and AT by flow cytometry analysis. As shown in Fig. 2 isolated cells were positive for CD90 and CD150 and negative for CD34 and CD45 surface markers. The homogeneity of isolated MSCs was more than 98%.

Characterization of differentiated brown and white adipocytes by Oil Red O staining

The light microscopy image of differentiated adipocytes from UCB-MSCs and AD-MSCs are shown in Fig. 3a–d. As seen in Fig. 3, UCB-MSCs were differentiated into both brown and white adipocytes with lower efficiency in comparison to AD-MSCs. Oil Red O staining was performed to stain accumulated lipids in cytoplasm of differentiated adipocytes (Fig. 3e–h). As shown in light microscopy and Oil Red O images, there was not significant differences between brown and white adipocytes differentiated from UCB-MSCs. Very small intracytoplasmic lipid droplets can be seen in adipocytes differentiated from UCB-MSCs. However, brown adipocytes differentiated form AD-MSCs contain smaller lipid vacuoles in comparison to white adipocytes.

The red pixel amount of each picture was quantified using ImageJ program. As shown in Fig. 3i, the maximum red pixel level was seen in white (85%) and brown (61%) adipocytes differentiated from AD-MSCs. White (3%) and brown adipocytes (1.9%) differentiated from UCB-MSCs had very low amount of red pixel. Since Oil Red O dissolves in lipids, the red pixel level showed the lipid amount accumulated in cytoplasm of adipocytes. As a result, the lowest oil amount and differentiation potential was seen in adipocytes differentiated from UCB-MSCs.

HE staining results

HE staining was performed to clarify morphology of differentiated adipocytes and investigate size and number of lipid vacuoles. There was not significant difference between white and brown adipocytes differentiated from UCB-MSCs in HE staining, as same as Oil Red O staining results (Fig. 4a, b). As shown in Fig. 4, the cytoplasm of differentiated brown adipocytes contains smaller lipid vacuoles than white adipocytes, especially in brown adipocytes differentiated form AD-MSCs. However, most of the white adipocytes differentiated form AD-MSCs contains low number huge lipid vacuoles while an excessive number of small lipid vacuoles can be seen in cytoplasm of brown adipocytes (Fig. 4c, d).

Immunofluorescence analysis of UCP1 protein

The UCP1 expression was used commonly as a recognition biomarker of brown adipocytes. In this study, the UCP1 protein was investigated in differentiated brown adipocytes by immunofluorescence analysis. As shown in Fig. 5, while UCP1 protein was so low in undifferentiated MSCs, brown adipocytes show strong fluorescence signal related to UCP1 protein. Especially, in brown adipocyte differentiated form AD-MSCs a very strong fluorescence signal can be seen in cytoplasm. Brown adipocytes from UCB-MSCs showed a little increase in fluorescent signal than the ones from AD-MSCs.

Western blot results

The UCP1 protein expression levels were investigated by western blot analysis. The quantification of UCP1 amounts was done using ImageJ program. The UCP1 protein expression levels were fourfold ($P < 0.01$) and 10.64-fold ($P < 0.003$) higher than undifferentiated MSCs (Fig. 6a, b), whereas white adipocytes showed approximately the same level of UCP1 protein. Brown adipocytes differentiated from AD-MSCs showed 2.5-fold increase in UCP1 protein levels when compared with those from UCB-MSCs.

Discussion

Recently, researchers intend to perform in vitro differentiation and transplantation of brown adipocytes as a possible cure of obesity and its related complications such as diabetes, cardio vascular diseases and cancer [20]. Therefore, performing more in vitro studies can help to optimize therapeutic protocols. In this study, the differentiation potential of MSCs from UCB and AT into brown and white adipocytes was compared. There is not any study in the literature investigating differentiation of UCB-MSCs into brown adipocytes until now.

In this study, approximately 300 ml abdominal liposuction materials collected from six healthy adult females, and 50 ml UCB samples collected from ten healthy pregnant donors having single gestation pregnancy, after full-term delivery. Both UCB and liposuction materials were processed immediately after collection to isolate MSCs. 2×10^8 mononuclear cells were isolated from 50 ml UCB samples

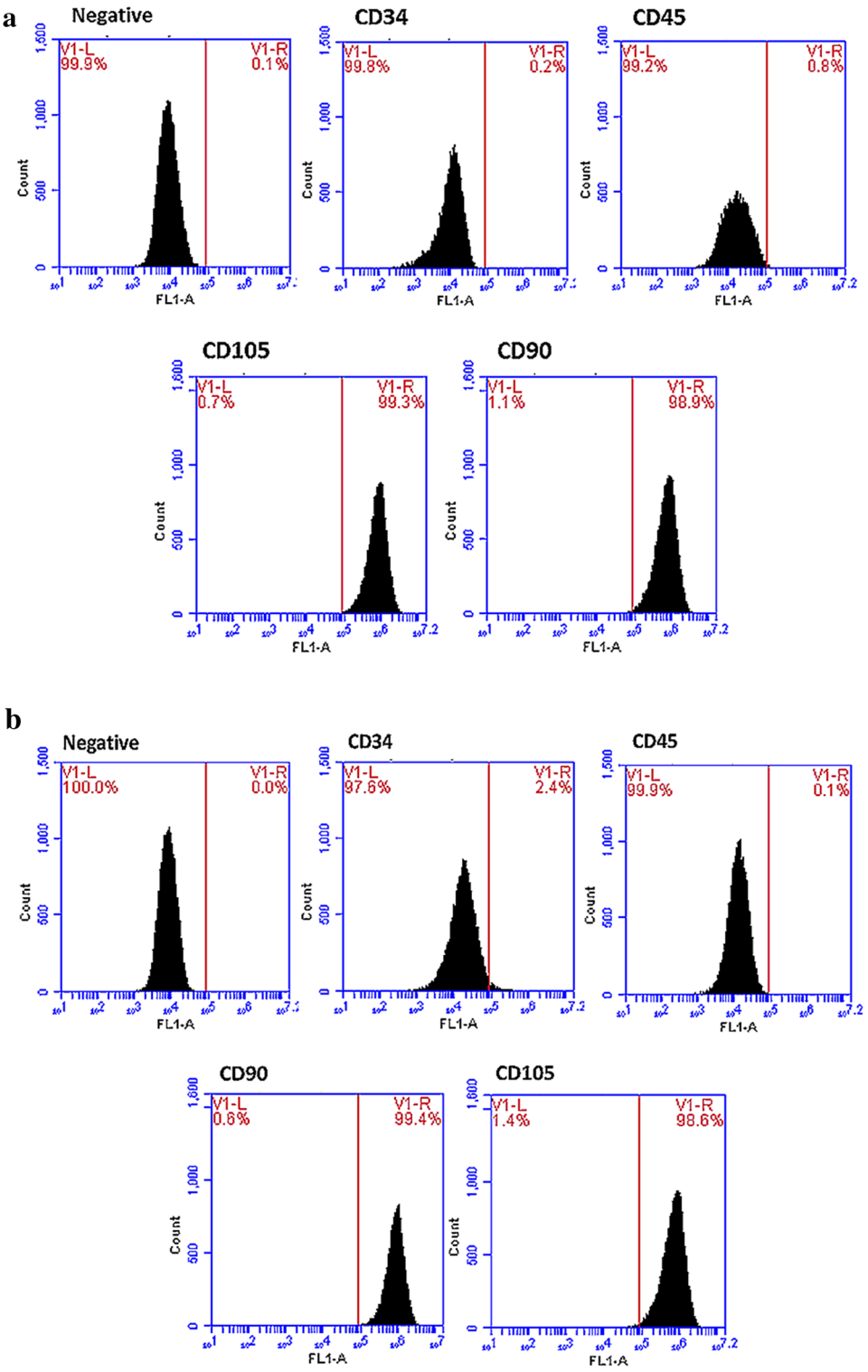

Fig. 2 Flow cytometry results. **a** UCB-MSCs, **b** AD-MSCs

by Ficoll gradient centrifugation method. The cell colonies were appeared at the end of the first week (Fig. 1a) and the fibroblastic cells appeared at the end of the second week. Five colonies were achieved from 2×10^8 mononuclear cells.

In 2003, Wexler et al. showed that the UCB is not a reliable source for isolation of MSCs, because its stem cell numbers are so low and they show hematopoietic characteristics [13]. Secco et al. showed isolation of MSCs from one sample out of 10 UCB samples (10% success) [21] while, Shetty et al. demonstrated MSCs isolation from only 6% of UCB samples [22]. However, this isolation success ratio was increased to 57 and 63% in the studies done by Fujii

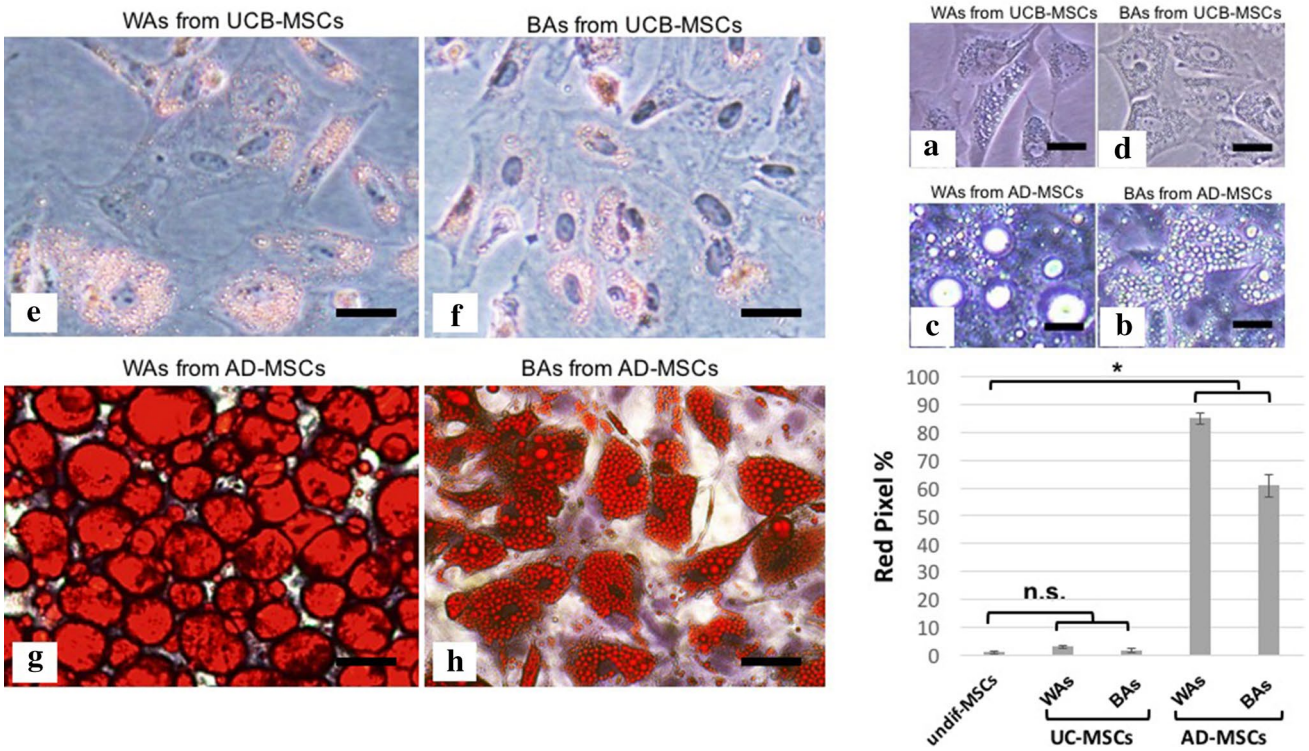

Fig. 3 Microscopy image of differentiated adipocytes and red pixel analysis (scale bar = 50 μM). **a**–**d** light microscopy images, and **e**–**h** Oil Red O stained images. **a** and **e** images show white adipocytes and b and f show brown adipocytes differentiated from UCB-MSCs. **c** and **g** images demonstrated white adipocytes and **d** and **h** show brown adipocytes differentiated from AD-MSCs (Objective 40×). **i** The red pixel percentage in differentiated cells. The red pixel demonstrated accumulated oil amount in cytoplasm of adipocytes and used as an indicator of adipocyte differentiation potential in this study. *WAs* white adipocytes, *BAs* brown adipocytes, Error bars showed SEM. *n.s.* not significant. *P < 0.001. (Color figure online)

et al. [23] and Kern et al. [24], respectively. Meanwhile, in concordance with these recent studies, fibroblastic cells have been isolated from 70% of UCB samples in our study. Moreover, according to established cell colonies in our study, one MSCs were isolated from each 4×10^7 mononuclear cells. These results are similar to the results of Bieback et al. who had reported 0–2.3 colonies from 1×10^8 mononuclear cells [11]. Based on instruction published in 2005 by The Committee of Mesenchymal Stem Cells and Tissues of the International Society for Cellular Therapy (ISCT) the MSCs statement can be used to define stem cells express CD105, CD73, and CD90/Thy-1 more than 95% and CD11b, CD14, CD19, CD34, CD45, CD79a, and HLA-DR surface markers less than or equal to 2%+ [25]. In present study, isolated stem cells form both AT and UCB expressed CD90, CD105 but not expressed hematopoietic surface antigens (CD34−, CD45−) (Fig. 2). The flow cytometry results presented in this study are also similar to the results of the studies recently done by Griffin et al. [26] and Choi et all [27].

According to the Sibov et al. the higher MSCs number can be isolated from UCB samples collected from normal full-term delivery with less than 37 weeks. In the other hand, the blood sample should be around 80 ml and must be stored in less than 6 h [28]. According to Bieback et al. the critical points in isolation of UCB-MSCs are collection of UCB samples in less than 15 h after delivery, collecting more than 33 ml blood sample, and isolating more than 1×10^8 mononuclear cells from each blood sample [11]. In this study, collecting 50 ml UCB sample immediately after full-term delivery, and processing them immediately without storage can be the reason for successful isolation of MSCs. We found that MSCs isolated from UCB samples show fibroblastic structure in the late stages (after 2 weeks culturing), and the first passage was done after 3 weeks (Fig. 1b, c). This means that, isolation of MSCs from UCB is challenging and time-consuming which requires a lot of patience because of low proliferation rate of these cells during isolation procedure. Choi et al. recently optimized the culturing condition via using Ca^{2+}/hypoxia which leads to an increase in the proliferation ratio and in the expression levels of *Oct4* and *Nanog* known as stemness genes in comparison to cells which were cultured in conventional conditions, without any change in their therapeutic efficiency [27]. As Choi et al. mentioned, the new culturing

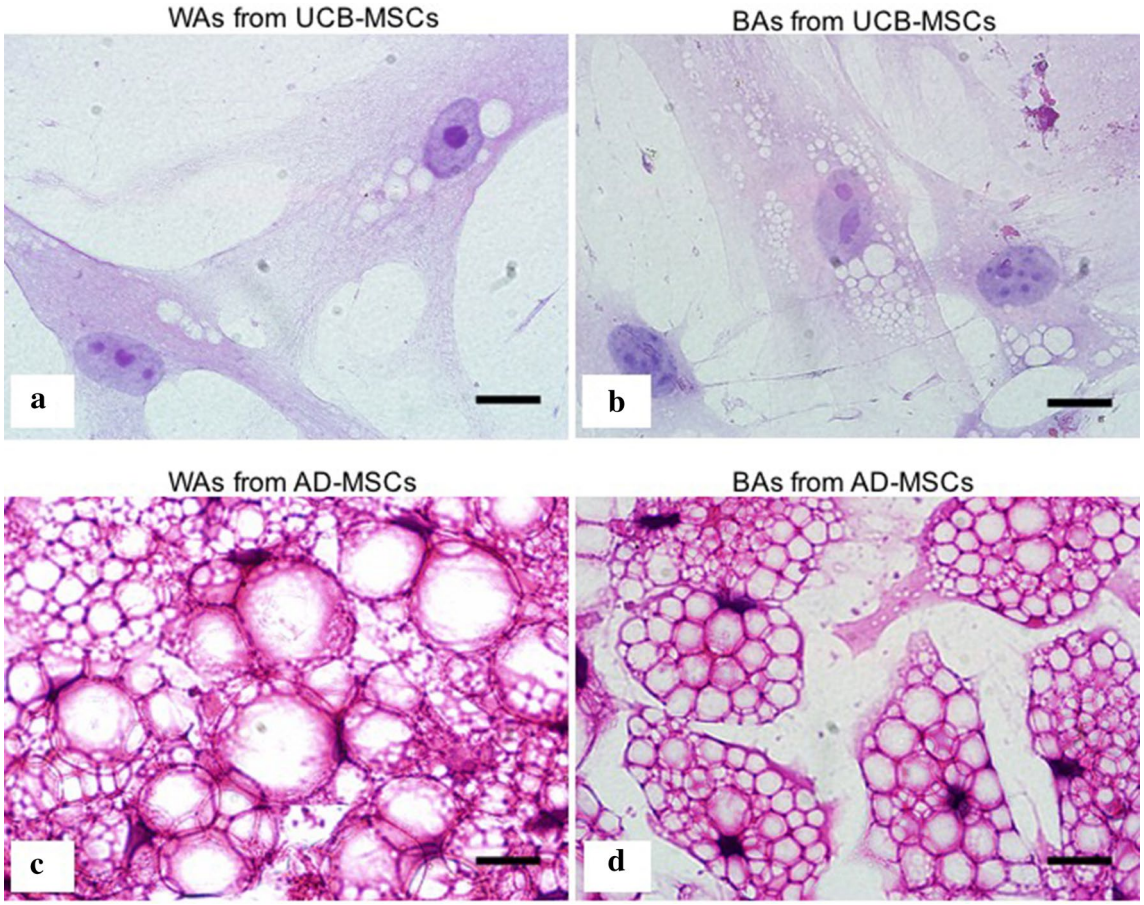

Fig. 4 HE staining of differentiated adipocytes (scale bar = 20 μM). There is not significant difference between white (**a**) and brown (**b**) adipocytes differentiated form UCB-MSCs. Most of the white adipocytes (**c**) differentiated from AD-MSCs contained just one huge lipid vacuole, but brown adipocytes (**d**) contained multiple small lipid vacuoles. *WAs* white adipocytes, *BAs* brown adipocytes. Objective 100×. (Color figure online)

condition may be useful to accelerate hUCB-MSCs expansion for clinical applications [27].

Approximately 1×10^9 cells with > 90% viability isolated from 300 ml liposuction materials. Cells were passaged at 7th day after incubation (Fig. 1d). MSCs isolated form all (100%) liposuction samples. In this regard, our results are also similar to the results of previous studies in the literature [29–31], in which Kern et al. [24], and Griffin et al. [26] were also able to show 100% successful isolation of MSCs from AT.

Because of the increased interest in the stem cell transplantation for soft tissue disorders and lacking any literature regarding UCB-MSCs differentiation into brown adipocytes we decided to compare the ability of UCB-MSCs with those derived from AT. According to the results of the present study, AD-MSCs showed high differentiation potential into both white (85% red pixel) and brown (61% red pixel) adipocytes while differentiation potential of UCB-MSCs into white and brown adipocytes was approximately 3 and 1.9% respectively (Fig. 3). HE staining revealed exact morphology of differentiated white and brown adipocytes (Fig. 4). In this study, the Oil Red O staining results of differentiated adipocytes from UCB-MSCs are similar to the results of Koch et al. related to white adipocytes differentiated from UCB-MSCs [32]. Rebelatto et al. recently compared the differentiation potential of AD-MSCs and UCB-MSCs into white adipocytes [33]. In accordance with the results of Rebelatto et al., our results show more adipogenic differentiation potential of AD-MSCs when compared to UCB-MSCs. We showed that white adipocytes differentiated form AD-MSCs are more natural and have a huge lipid vacuole in their cytoplasm. For the first time, in this study we investigated the differentiation potential of UCB-MSCs into brown adipocytes. Based on our results, UCB-MSCs cannot be efficiently differentiated into brown adipocytes. In the literature, Kern et al. who showed differentiation of AD-MSCs into white adipocytes with 94% successfully, but, was not able to differentiate UCB-MSCs into white adipocytes even after incubation in differentiating medium for 5 weeks [24]. They reported lower differentiation potential of UCB-MSCs to

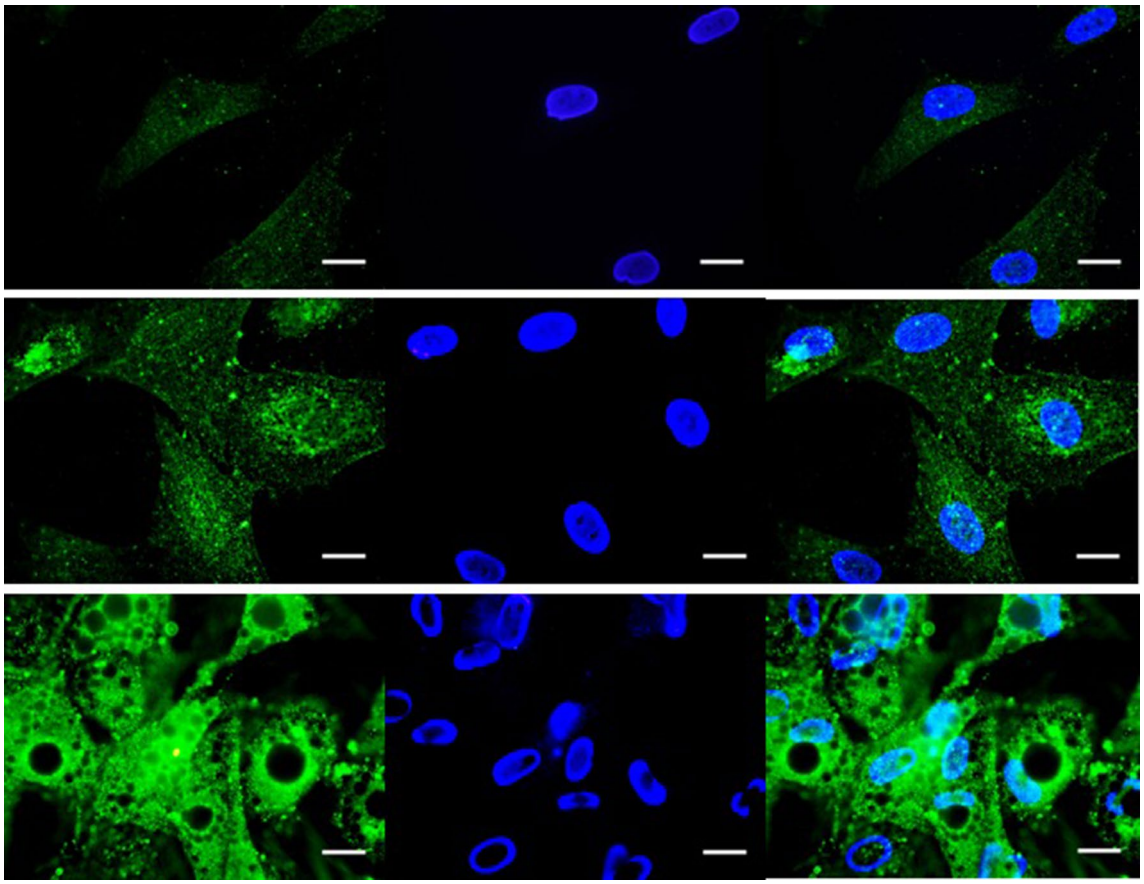

Fig. 5 The immunofluorescence results of UCP1 protein staining (scale bar = 20 μM). **a** Undifferentiated MSCs as a control, **b** brown adipocytes differentiated form UCB-MSCs, **c** brown adipocytes differentiated form AD-MSCs. objective 100×. (Color figure online)

white adipocytes when compared with AD-MSCs or BM-MSCs [24]. Similarly, Shetty et al. have stated that UCB-MSCs did not show any lipid vacuoles after trying several experiments for adipogenic differentiation [22]. Contrary to Shetty et al. who failed to obtain adipogenic differentiation of UCB-MSCs, in our study we managed adipogenic differentiation of UCB-MSCs which were confirmed with the small lipid vacuoles seen in both white and Brown adipocytes differentiated from UCB-MSCs as shown in Figs. 3e, f and 4 a, b. However, according to our results, it can be concluded that AD-MSCs can be differentiated into both mature brown and white adipocytes with higher efficiency than UCB-MSCs as it was confirmed with our results both via immunofluorescence and western blot analysis (Figs. 5, 6a). Fluorescence microscopy results showed more strong signals related to UCP1 protein in brown adipocytes in comparison to undifferentiated MSCs, especially for that which were differentiated form AD-MSCs (Fig. 5). Western blot results showed the 10.64-fold increase of UCP1 protein levels in brown adipocytes from AD-MSCs ($P < 0.003$), while only fourfold increase was detected in brown adipocytes derived from UCB-MSCs ($P < 0.01$) (Fig. 6a, b, respectively). This means that, the UCP1 amount in brown adipocytes differentiated from AD-MSCs was 2.5 times more than that differentiated from UCB-MSCs. Our western blot results were also similar to the results of Xue et al. [34] and Guénantin et al. [35].

As shown by Schulz et al. [36], and Xue et al. [37] in the presence of BMP7, AD-MSCs can be differentiated into brown adipocytes with high efficiency. Especially, BMP7 can increase the UCP1 protein expression around 9 times when it is used along with T3 and Indomethacin. BMP7 induce differentiation of stem cells into brown adipocytes and trigger the thermogenic program [37]. These results are all in concordance with our results and highlight that BMP7 has a positive effect in increasing cellular thermogenesis by inducing UCP1 gene expression, which is specific to brown adipocytes, and also in reducing fat storage in differentiated brown adipocytes when compared to white adipocytes (Figs. 3, 5, 6). In different study, Zhang et al. recently investigated irisin effects on browning of human mature white adipocytes differentiated from preadipocytes [38]. These researchers showed an increase in UCP1 protein levels up to threefold in brown adipocytes differentiated from mature

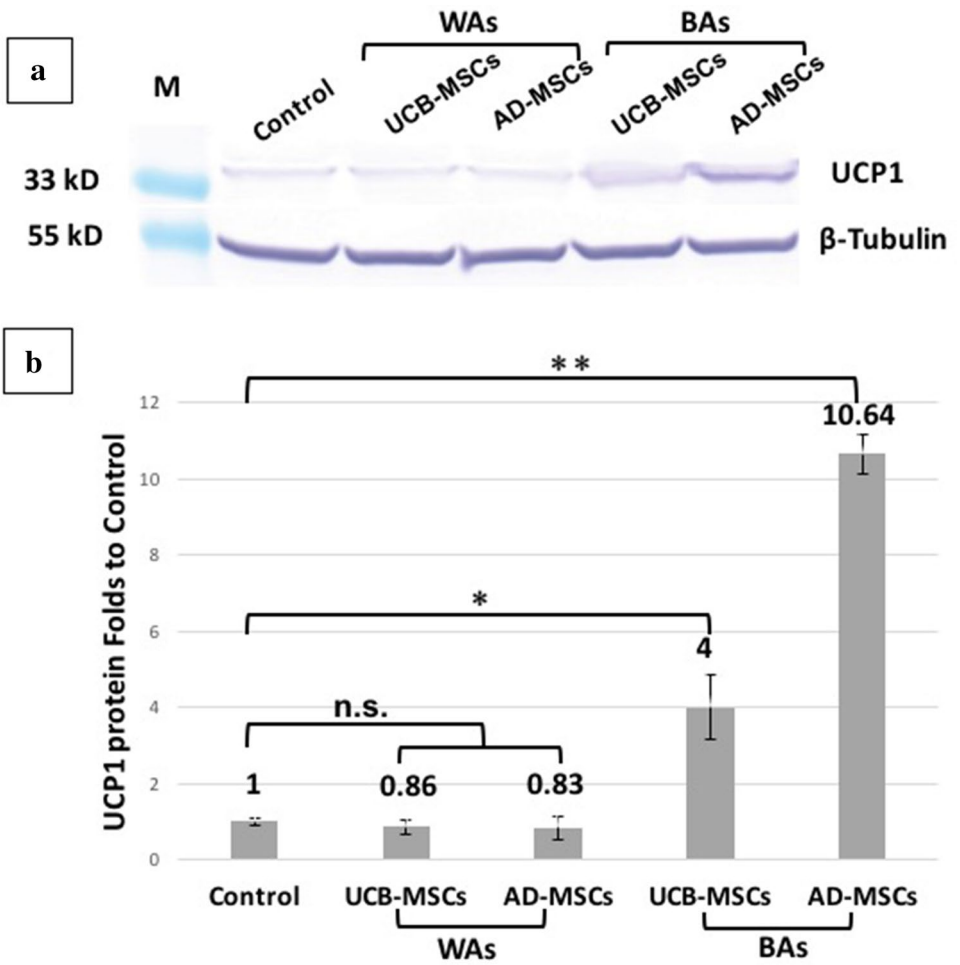

Fig. 6 Western blot results. **a** Protein bands related to UCP1 and β-tubulin. **b** Quantification of UCP1 amounts. The UCP1 protein levels were 4- and 10.64-fold higher than the levels in undifferentiated MSCs. *WAs* white adipocytes, *BAs* brown adipocytes. Error bars demonstrate SEM. *n.s.* not significant. *P < 0.01, **P < 0.003. (Color figure online)

primary adipocytes after 4-day treatment with irisin by western blot and IF analysis [38] in comparison to the control groups.

Although, we showed the low efficiency of UCB-MSCs in differentiation into brown and white adipocytes in comparison to AD-MSCs, it doesn't mean that these cells cannot be differentiated into other cell types. In this regard, the differentiation potential of UC-MSCs into various cell types was investigated by different groups. In the retrospective study performed by Abo-Elkheir et al. the efficiency of UCB- and BM-MSCs in deep burns was investigated [39]. Although, the authors concluded that transplanted MSCs from both sources, as bone marrow and cord blood, effectively improved healing of burn injuries, hyperpigmentation, hypertrophic scar formation, and early complications such as infection and partial loss of graft, were higher in patients who were treated with UCB-MSCs in comparison to BM-MSCs. In contrast, the amount of contractured scar formation (10%) was equal in both UCB- and BM-MSCs-transplanted groups, and the hypopigmentation ratio was less in UCB-MSCs-transplanted group [39]. Recently, Yousefi et al. showed expression of motor neuron- related markers after in vitro differentiation of UCB-MSCs into motor neuron-like cells [40] while other groups succeeded in differentiation of UCB-MSCs into the hair follicle cells [41], insulin-producing cells [42], and hepatocytes [43]. Therefore, further studies are needed to prefigure potential tendency of each UCB- and AD-MSCs for differentiation into the specific cell types with the overall goal of developing new cell based therapies for specific disorders.

Consequently, UCB can be used as a stem cell isolation source as same as liposuction materials. Nevertheless, the stem cell number of UCB is lower than AT and isolation of MSCs require longer culturing periods. AD-MSCs can be differentiated into brown and white adipocytes with higher efficiency in comparison to UCB-MSCs. Western blot and immunofluorescence analysis showed significant increase of UCP1 protein in brown adipocytes differentiated from AD-MSCs, whereas, the differentiation efficiency of UCB-MSCs into both brown and white adipocytes was so low. According to the results of this study, we believe that AD-MSCs are suitable stem cell type for in vitro and in vivo differentiation into brown adipocytes, aiming to find a new therapeutic

approach for obesity and its related complications. In this regard, further in vivo and clinical trials are needed.

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Compliance with ethical standards

Conflicts of interest The authors declared that they have no conflict of interest.

Ethical standards Research involved human participants and all procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Informed consent Informed consent was obtained from all individual participants included in the study

Research involving with human and animals participants This article does not contain any studies with animals performed by any of the authors.

References

1. Farese RV, Zechner R, Newgard CB et al (2012) The problem of establishing relationships between hepatic steatosis and hepatic insulin resistance. Cell Metab 15:570–573. https://doi.org/10.1016/j.cmet.2012.03.004
2. Langstein HN, Robb GL (1999) Reconstructive approaches in soft tissue sarcoma. Semin Surg Oncol 17:52–65
3. Chen YJ, Chen CC, Li TK et al (2012) Docosahexaenoic acid suppresses the expression of FoxO and its target genes. J Nutr Biochem 23:1609–1616. https://doi.org/10.1016/j.jnutbio.2011.11.003
4. Chen YJ, Liu HY, Chang YT et al (2016) Isolation and differentiation of adipose-derived stem cells from porcine subcutaneous adipose tissues. J Vis Exp 109:e53886. https://doi.org/10.3791/53886
5. Gharbia O, Afify AE, Abd El Ghaffar H et al (2015) The regenerative effect of human umbilical cord blood mesenchymal stem cells in a rabbit model of osteoarthritis. Egypt Rheumatol Rehabil 42:163–169. https://doi.org/10.4103/1110-161X.168151
6. Fallahi-Sichani M, Soleimani M, Najafi SM et al (2007) In vitro differentiation of cord blood unrestricted somatic stem cells expressing dopamine-associated genes into neuron-like cells. Cell Biol Int 31:299–303. https://doi.org/10.1016/j.cellbi.2006.11.011
7. Cohena Y, Nagler A (2003) Hematopoietic stem-cell transplantation using umbilical-cord blood. Leuk Lymphoma 44:1287–1299
8. Ooi J (2006) The efficacy of unrelated cord blood transplantation for adult myelodysplastic syndrome. Leuk Lymphoma 47:599–602. https://doi.org/10.1080/10428190500421013
9. Laughlin MJ, Barker J, Bamback B et al (2001) Hematopoietic engraftment and survival in adult recipients of umbilical-cord blood from unrelated donors. New Engl J Med 344:1815–1822. https://doi.org/10.1056/NEJM200106143442402
10. Lee OK, Kuo TK, Chen WM et al (2004) Isolation of multipotent mesenchymal stem cells from umbilical cord blood. Blood 103:1669–1675. https://doi.org/10.1182/blood-2003-05-1670
11. Bieback K, Kern S, Kluter H et al (2004) Critical parameters for the isolation of mesenchymal stem cells from umbilical cord blood. Stem Cells 22:625–634. https://doi.org/10.1634/stemcells.22-4-625
12. Mareschi K, Biasin E, Piacibello W et al (2001) Isolation of human mesenchymal stem cells: bone marrow versus umbilical cord blood. Haematologica 86:1099–1100
13. Wexler SA, Donaldson C, Denning-Kendall P et al (2003) Adult bone marrow is a rich source of human mesenchymal 'stem' cells but umbilical cord and mobilized adult blood are not. Br J Haematol 121:368–374
14. Okla M, Ha JH, Temel RE et al (2015) BMP7 drives human adipogenic stem cells into metabolically active beige adipocytes. Lipids 50:111–120. https://doi.org/10.1007/s11745-014-3981-9
15. Elsen M, Raschke S, Tennagels N et al (2014) BMP4 and BMP7 induce the white-to-brown transition of primary human adipose stem cells. Am J Physiol Cell Physiol 306:C431–C440. https://doi.org/10.1152/ajpcell.00290.2013
16. Yuan SH, Bi Z (2012) Effect of recombinant adeno-associated BMP-4/7 fusion gene on the biology of BMSCs. Mol Med Rep 6:1413–1417. https://doi.org/10.3892/mmr.2012.1090
17. Nishio M, Yoneshiro T, Nakahara M et al (2012) Production of functional classical brown adipocytes from human pluripotent stem cells using specific hemopoietin cocktail without gene transfer. Cell Metab 16:394–406. https://doi.org/10.1016/j.cmet.2012.08.001
18. Ahfeldt T, Schinzel RT, Lee YK et al (2012) Programming human pluripotent stem cells into white and brown adipocytes. Nat Cell Biol 14:209–219. https://doi.org/10.1038/ncb2411
19. Zhu M, Heydarkhan-Hagvall S, Hedrick M et al (2013) Manual isolation of adipose-derived stem cells from human lipoaspirates. J Vis Exp 26:e50585. https://doi.org/10.3791/50585
20. Kopecky J, Clarke G, Enerbäck S et al (1995) Expression of the mitochondrial uncoupling protein gene from the aP2 gene promoter prevents genetic obesity. J Clin Invest 96:2914–2923. https://doi.org/10.1172/JCI118363
21. Secco M, Zucconi E, Vieira NM et al (2008) Multipotent stem cells from umbilical cord: cord is richer than blood. Stem Cells 26:146–150. https://doi.org/10.1634/stemcells.2007-0381
22. Shetty P, Cooper K, Viswanathan C (2010) Comparison of proliferative and multilineage differentiation potentials of cord matrix, cord blood, and bone marrow mesenchymal stem cells. Asian J Transfus Sci 4:14–24. https://doi.org/10.4103/0973-6247.59386
23. Fujii S, Miura Y, Iwasa M et al (2017) Isolation of mesenchymal stromal/stem cells from cryopreserved umbilical cord blood cells. J Clin Exp Hematopathol 57:1–8. https://doi.org/10.3960/jslrt.16019
24. Kern S, Eichler H, Stoeve J et al (2006) Comparative analysis of mesenchymal stem cells from bone marrow, umbilical cord blood, or adipose tissue. Stem Cells 24:1294–1301. https://doi.org/10.1634/stemcells.2005-0342
25. Ledesma-Martínez E, Mendoza-Núñez VM, Santiago-Osorio E (2016) Mesenchymal stem cells derived from dental pulp: a review. Stem Cells Int 2016:4709572. https://doi.org/10.1155/2016/4709572
26. Griffin M, Ryan CM, Pathan O et al (2017) Characteristics of human adipose derived stem cells in scleroderma in comparison to sex and age matched normal controls: implications for regenerative medicine. Stem Cell Res Ther 8:23. https://doi.org/10.1186/s13287-016-0444-7
27. Choi W, Kwon SJ, Jin HJ et al (2017) Optimization of culture conditions for rapid clinical-scale expansion of human umbilical cord blood-derived mesenchymal stem cells. Clin Transl Med 6:38. https://doi.org/10.1186/s40169-017-0168-z
28. Sibov TT, Severino P, Marti LC et al (2012) Mesenchymal stem cells from umbilical cord blood: parameters for isolation, characterization and adipogenic differentiation. Cytotechnology 64:511–521. https://doi.org/10.1007/s10616-012-9428-3

29. Zuk PA, Zhu M, Mizuno H et al. (2001) Multilineage cells from human adipose tissue: implications for cell-based therapies. Tissue Eng 7:211–228. https://doi.org/10.1089/107632701300062859
30. Aust L, Devlin B, Foster SJ et al (2004) Yield of human adipose-derived adult stem cells from liposuction aspirates. Cytotherapy 6:7–14. https://doi.org/10.1080/14653240310004539
31. Boquest AC, Shahdadfar A, Brinchmann JE et al (2006) Isolation of stromal stem cells from human adipose tissue. Methods Mol Biol 325:35–46. https://doi.org/10.1385/1-59745-005-7:35
32. Koch TG, Heerkens T, Thomsen PD et al (2007) Isolation of mesenchymal stem cells from equine umbilical cord blood. BMC Biotechnol 7:26. https://doi.org/10.1186/1472-6750-7-26
33. Rebelatto CK, Aguiar AM, Moretão MP et al (2008) Dissimilar differentiation of mesenchymal stem cells from bone marrow, umbilical cord blood, and adipose tissue. Exp Biol Med 233:901–913. https://doi.org/10.3181/0712-RM-356
34. Xue R, Lynes MD, Dreyfuss JM et al (2015) Clonal analyses and gene profiling identify genetic biomarkers of the thermogenic potential of human brown and white preadipocytes. Nat Med 21:760–768. https://doi.org/10.1038/nm.3881
35. Guénantin AC, Briand N, Capel E et al (2011) Functional human beige adipocytes from induced pluripotent stem cells. Diabetes 66:1470–1478. https://doi.org/10.2337/db16-1107
36. Schulz TJ, Huang TL, Tran TT et al (2011) Identification of inducible brown adipocyte progenitors residing in skeletal muscle and white fat. PNAS 108:143–148. https://doi.org/10.1073/pnas.1010929108
37. Xue R, Wan Y, Zhang S et al (2014) Role of bone morphogenetic protein 4 in the differentiation of brown fat-like adipocytes. Am J Physiol Endocrinol Metab 306:E363–E372. https://doi.org/10.1152/ajpendo.00119.2013
38. Zhang Y, Xie C, Wang H et al (2016) Irisin exerts dual effects on browning and adipogenesis of human white adipocytes. Am J Physiol Endocrinol Metab 311:E530–E541. https://doi.org/10.1152/ajpendo.00094.2016
39. Abo-Elkheir W, Hamza F, Elmofty AM et al (2017) Role of cord blood and bone marrow mesenchymal stem cells in recent deep burn: a case-control prospective study. Am J Stem Cells 6:23–35
40. Yousefi B, Sanooghi D, Faghihi F et al (2017) Evaluation of motor neuron differentiation potential of human umbilical cord blood-derived mesenchymal stem cells, in vitro. J Chem Neuroanat 81:18–26. https://doi.org/10.1016/j.jchemneu.2017.01.003
41. Bu ZY, Wu LM, Yu XH et al (2017) Isolation and characterization of in vitro culture of hair follicle cells differentiated from umbilical cord blood mesenchymal stem cells. Exp Ther Med 14:303–307. https://doi.org/10.3892/etm.2017.4456
42. Van Pham P, Thi-My Nguyen P, Thai-Quynh Nguyen A et al (2014) Improved differentiation of umbilical cord blood-derived mesenchymal stem cells into insulin-producing cells by PDX-1 mRNA transfection. Differentiation 87:200–208. https://doi.org/10.1016/j.diff.2014.08.001
43. Tang XP, Zhang M, Yang X et al (2006) Differentiation of human umbilical cord blood stem cells into hepatocytes in vivo and in vitro. World J Gastroenterol 12:4014–4019

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