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Enhanced Adipogenic Differentiation of Human Adipose-Derived Stem Cells in an In Vitro Microenvironment: The Preparation of Adipose-Like Microtissues Using a Three-Dimensional Culture

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The application of stem cells for cell therapy has been extensively studied in recent years. Among the various types of stem cells, human adipose tissue-derived stem cells (ASCs) can be obtained in large quantities with relatively few passages, and they possess a stable quality. ASCs can differentiate into a number of cell types, such as adipose cells and ectodermal cells. We therefore focused on the in vitro microenvironment required for such differentiation and attempted to induce the differentiation of human stem cells into microtissues using a microelectromechanical system. We first evaluated the adipogenic differentiation of human ASC spheroids in a three-dimensional (3D) culture. We then created the in vitro microenvironment using a 3D combinatorial TASCL device and attempted to induce the adipogenic differentiation of human ASCs. The differentiation of human ASC spheroids cultured in maintenance medium and those cultured in adipocyte differentiation medium was evaluated via Oil red O staining using lipid droplets based on the quantity of accumulated triglycerides. The differentiation was confirmed in both media, but the human ASCs in the 3D cultures contained higher amounts of triglycerides than those in the 2D cultures. In the short culture period, greater adipogenic differentiation was observed in the 3D cultures than in the 2D cultures. The 3D culture using the TASCL device with adipogenic differentiation medium promoted greater differentiation of human ASCs into adipogenic lineages than either a 2D culture or a culture using a maintenance medium. In summary, the TASCL device created a hospitable in vitro microenvironment and may therefore be a useful tool for the induction of differentiation in 3D culture. The resultant human ASC spheroids were "adipose-like microtissues" that formed spherical aggregation perfectly and are expected to be applicable in regenerative medicine as well as cell transplantation.

Key words: Adipose-derived stem cells (ASCs); Spheroid; Three-dimensional (3D) culture; Tapered stencil for cluster culture (TASCL); Adipogenic differentiation; Microenvironment

INTRODUCTION

A large number of studies in recent years have focused on stem cells and their role in cell therapy. Stem cells are cells with self-renewal and differentiation capabilities, and several different types have been identified, each with the potential to differentiate into specialized cell types, such as embryonic stem cells (ESCs)[1] and induced pluripotent stem cells (iPSCs)[2,3] with pluripotency, bone marrow-derived mesenchymal stem cells and mesenchymal stem cells (BM-MSCs and MSCs, respectively)[4,5], adipose tissue-derived stem cells (ASCs)[6,7], and other tissue-derived stem cells with multipotency.

Among these cell types, human ASCs can be obtained from human adipose tissues via relatively low-invasive procedures. Furthermore, human ASCs can be obtained in large quantities over relatively few passages and in high quality with respect to their proliferation and differentiation abilities. Previous reports have cited the high quality of cryopreserved human ASCs[8–10], which are an important source of cells for cell transplantation[11–13] and

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regenerative medicine. ASCs can differentiate into a number of cell types, such as endodermal[14], mesodermal[15], and ectodermal cells[16]. However, the effective use of human ASCs will require ensuring their function is maintained, including their self-renewal and differentiation abilities.

To maintain stem cells in an undifferentiated state, we need to optimize the culture conditions. The methods for maintaining stem cells in an undifferentiated state in two-dimensional (2D) culture are well established[17], but such methods for 3D culture are still being developed, although a few reports[18–22] have been published on the matter. Controlling the induction of differentiation into target cells is important, as is the maintenance of stemness. The induction of differentiation is essential in the application of these cells to cell therapy and regenerative medicine. In cells with substantial differentiation capacity, such as human ESCs and iPSCs, the targeted induction of differentiation into specific cells is difficult in both 2D and 3D cultures[23,24]. In contrast, human tissue-derived stem cells, such as human MSCs[4,5] and ASCs[7,25], retain their multipotency, and their differentiation is much easier to control in both 2D and 3D cultures. Other factors potentially involved in controlling the differentiation of stem cells include the chemical compounds, low-molecular weight compounds, proteins, nucleic acids, genes, and the culture environment conditions (e.g., levels of O_2 and CO_2) used for the culture.

Previous reports have underscored the importance of cell–cell interactions in keeping stem cells undifferentiated and in inducing their differentiation into target cells in vitro[4,5,24,26–28]. The controlled differentiation of stem cells is commonly used in 3D culture, with microenvironments created using a variety of biomaterials and microelectromechanical systems. Creating organs and tissues using stem cells can be difficult in 2D culture. However, 3D culture using stem cells and tissue cells can reliably reproduce organs and tissues in vitro[29–36]. We therefore focused on the in vitro microenvironment and attempted to differentiate human stem cells into microtissues using a microelectromechanical system.

We previously proposed the use of the tapered stencil for cluster culture (TASCL) method (Fig. 1A), an array employing tapered microapertures of varying sizes made of poly(dimethylsiloxane) (PDMS)[37,38], compared with 2D culture (Fig. 1B). We were able to use the combinatorial TASCL device to create multiple 3D cell spheroids under several different controlled seeding conditions simultaneously[33]. The TASCL device can be used quickly and simply and is useful for preparing hepatocyte spheroids[33,39]. In addition, we prepared uniform embryoid bodies (EBs) from iPSCs on a large scale using the TASCL device and thereby induced the differentiation of EBs into liver cells[40].

We herein report the application of the TASCL device to prepare human ASCs in vitro. The TASCL device will be used to prepare 3D microtissues for cell and tissue transplantation. A 256-microwell 10-mm×10-mm TASCL device was created, with a top aperture of 400×400, 600×600, and 800×800 μm and bottom diameter of 140, 180, 240, and 280 μm per microwell, respectively. A 60-mm culture dish with an ultralow cell attachment surface under the TASCL device was used in the 3D microtissue preparation. We evaluated the rates of spheroid formation and adipogenic differentiation of the human ASCs in the TASCL device via Oil red O staining using lipid droplets based on the quantity of accumulated triglycerides.

MATERIALS AND METHODS

Materials

Maintenance medium (#PM-1; preadipocyte medium) and adipocyte differentiation medium (#DM-2) were purchased from Zen-Bio, Inc. (Research Triangle Park, NC, USA). Dulbecco's phosphate-buffered saline without Ca^{2+} and Mg^{2+} supplementation (D-PBS-free) was purchased from Life Technologies Inc. (Carlsbad, CA, USA). Oil red O reagent and formaldehyde were obtained from Sigma-Aldrich (St. Louis, MO, USA). All other materials and chemicals not specified above were of the highest grade available.

Cells

Cryopreserved human ASCs were obtained from Zen-Bio, Inc. The cryopreserved cells [#ASC-F, Lot ASC062801; sex/age/body mass index (average)/number of patients: female/37/23.29/1] at passage 2 were purchased from Dainippon Sumitomo Pharma (Osaka, Japan).

Preparation of a Combinatorial TASCL Device

These procedures were performed in accordance with a previously reported protocol[33,37,38]. Briefly, a combinatorial TASCL device was created using PDMS (SYLGARD® 184; Dow Corning, Midland, MI, USA) and coated with an aqueous solution of poly(ethylene oxide)–poly(propylene oxide)–poly(ethylene oxide) triblock copolymer [1% (w/w) Anti-Link; Allvivo Vascular, Inc., Lake Forest, CA, USA] to prevent cell adhesion. After the unconjugated copolymers were washed with D-PBS-free, a TASCL device with a hydrophilic layer was obtained.

The TASCL device can be easily installed on a 60-mm culture dish with an ultralow cell attachment surface (Corning Inc., Corning, NY, USA) using tweezers (Fig. 2A). The TASCL device has an overall size of 10 mm×10 mm, with a thickness of 0.55 mm, including microwells with a top aperture of 400×400, 600×600, and 800×800 μm and bottom diameter of 140, 180, 240, and 280 μm per microwell (Fig. 2B). A scanning electron microscope (SEM) was used to visualize the TASCL device. Carbon tape (Nissin Enamel Manufacturing Co.

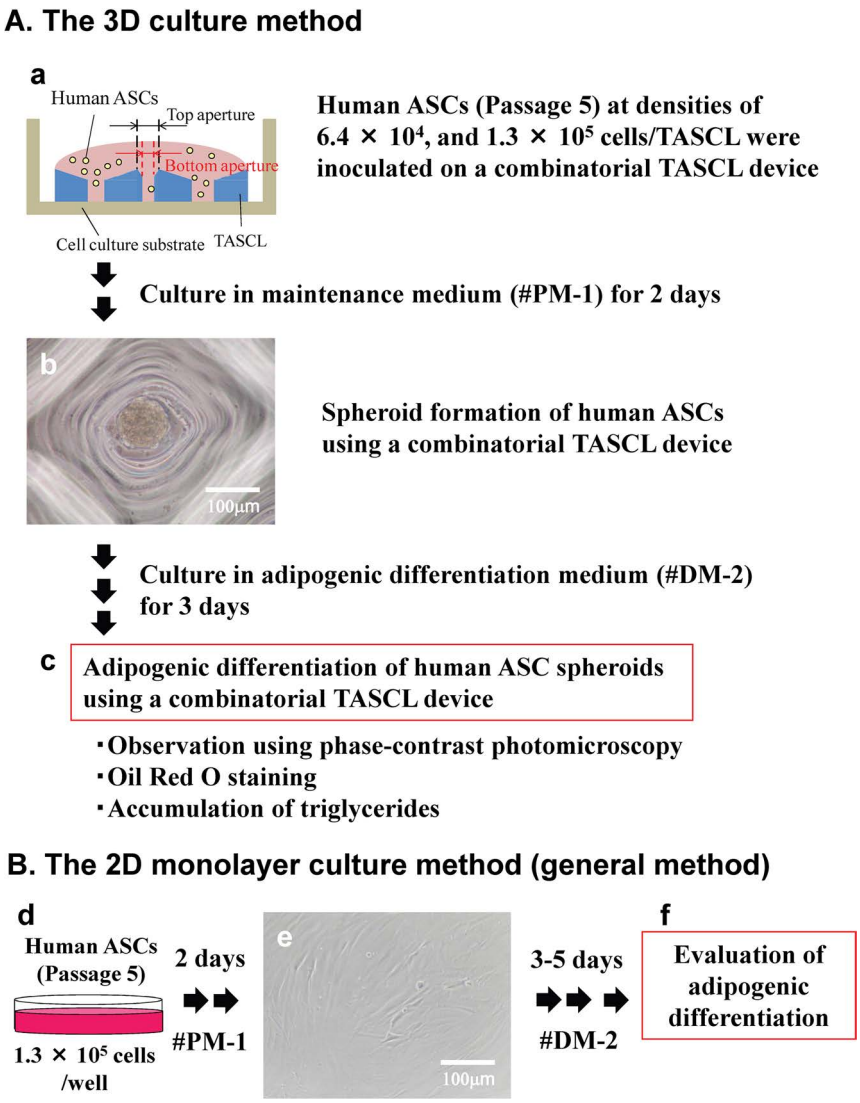

Figure 1. The experimental procedure for adipogenic differentiation induction of human adipose tissue-derived stem cells (ASCs) in 3D and 2D cultures. (A) Adipogenic differentiation of human ASCs in the 3D combinatorial tapered stencil for cluster culture (TASCL) device. (a) The TASCL device consisted of microwells measuring 10 mm×10 mm with a thickness of 0.55 mm. Each microwell in the TASCL device had a top aperture (400×400, 600×600, and 800×800 μm) and bottom aperture (140, 180, 240, and 280 μm in diameter). Human ASCs at a density of 6.4×10^5 or 1.3×10^5 cells/device were seeded onto the TASCL device in a maintenance medium and then were cultured for 2 days. (b) Spheroid formation of human ASCs in a microwell of the TASCL device. Human ASCs at a density of 1.3×10^5 cells/device were inoculated in maintenance medium for 2 days. Scale bar: 100 μm. (c) Evaluation of adipogenic differentiation of human ASC spheroids after 3 days of culture in adipogenic differentiation medium. Adipogenic differentiation of the human ASCs in the TASCL device was determined via Oil red O staining using lipid droplets based on the quantity of accumulated triglycerides. (B) Adipogenic differentiation of human ASCs in the 2D monolayer culture. (d) Human ASCs at a density of 1.3×10^5 cells/well were seeded onto a 12-well plate in maintenance medium and cultured for 2 days. (e) Phase-contrast image of the human ASCs after 2 days of culture. Scale bar: 100 μm. (f) Adipogenic differentiation of the human ASCs in the 2D monolayer culture was evaluated.

Ltd., Nagoya, Japan) was placed on the SEM stage (JEOL Ltd., Tokyo, Japan), and the TASCL device was attached to the tape. The shape of the TASCL device was confirmed using a field emission SEM (FE-SEM; JEOL JSM-7500F; JEOL Ltd.) at an acceleration voltage of 15 kV (Fig. 2C and D).

Thawing and Subculture of Human ASCs

For thawing, 2×10^5 human ASCs were seeded into a T-25 culture flask (NUNC, Roskilde, Denmark) in maintenance medium. The seeded cells attached and spread on the flask and were cultured at 37°C under a humidified 5% CO_2 atmosphere to approximately 80%–90%

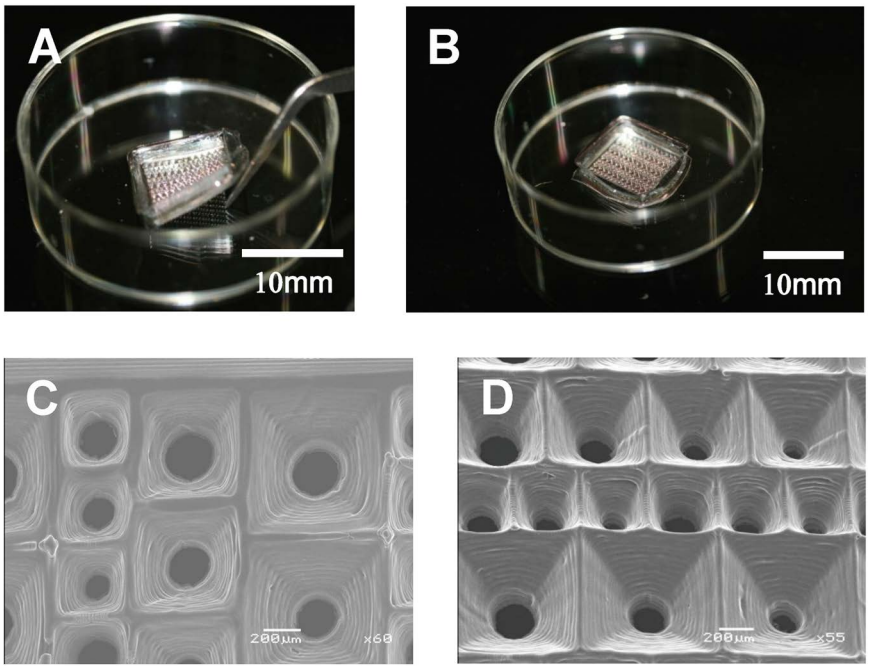

Figure 2. Scanning electron microscope (SEM) images of the combinatorial TASCL device. (A) The TASCL device can be easily installed on a 60-mm culture dish with an ultralow cell attachment surface with tweezers. (B) The gross appearance of the TASCL device consisted of microwells measuring 10 mm×10 mm with a thickness of 0.55 mm. Each microwell in the TASCL device had a top aperture (400×400, 600×600, and 800×800 μm) and bottom aperture (140, 180, 240, and 280 μm in diameter). Scale bars: 10 mm. (C, D) SEM image of a microwell in the TASCL device. Scale bars: 200 μm.

confluence. The cells were then detached from the flask by trypsin (Gibco®; Life Technologies Inc.) treatment and seeded and cultured in a new flask. The cell viability was more than 95%, as determined using the trypan blue dye exclusion test. The final concentration of trypan blue (Gibco®; Life Technologies Inc.) was 0.2%. The cells were passaged two times prior to use in the culture experiments with the TASCL device.

Adipogenic Differentiation of Human ASCs in 3D Culture

Figure 1A shows the experimental procedure of the spheroid formation and adipogenic differentiation induction of human ASCs using the combinatorial TASCL device (3D culture). Human ASCs at passage 5 were inoculated onto the TASCL device at densities of 6.4×10^4 and 1.3×10^5 cells/device to form 3D spheroids. The cells were then cultured in 0.35 ml of maintenance medium for 2 days at 37°C under a humidified 5% CO_2 atmosphere. The maintenance medium was partially changed every day. The morphology of human ASC spheroids was observed using a phase-contrast microscope (Olympus, Tokyo, Japan).

Adipogenic differentiation was induced by culturing human ASCs in adipocyte differentiation medium. In the control experiments, human ASC spheroids were cultured in maintenance medium, which was partially changed every day. After the cells had been cultured for 3 days using the TASCL device, they were stained to determine the accumulation of triglycerides by Oil red O staining, as described below. The morphology of the adipogenic differentiated human ASCs spheroids was observed under a phase-contrast microscope.

Adipogenic Differentiation of Human ASCs in 2D Culture

Figure 1B shows the experimental procedure of the monolayer culture and adipogenic differentiation induction of human ASCs in 2D culture. Human ASCs at passage 5 were inoculated onto 12-well plates (BD Falcon, Franklin Lakes, NJ, USA) at densities of 1.3×10^5 cells in 1 ml of maintenance medium. The cells were incubated at 37°C under a humidified 5% CO_2 atmosphere, and the medium was changed every day. The morphology of the human ASC monolayer was observed using a phase-contrast microscope. The adipogenic differentiation was performed as described above.

Evaluation of Adipogenic-Differentiated Human ASCs

The adipogenic differentiation of human ASCs was confirmed by microscopic observation of intracellular lipid droplets and Oil red O staining as an indicator of

the intracellular lipid accumulation. Briefly, differentiated human ASCs were fixed with 10% formaldehyde in D-PBS-free for 10 min at room temperature and washed with 60% isopropanol (Wako Pure Chemical Industries, Ltd., Osaka, Japan). The cells were then stained with 2% (w/v) Oil red O reagent for 10 min at room temperature followed by repeated washing with distilled water and then destained in isopropanol for 1 min. The Triglyceride E-test™ assay (Wako Pure Chemical Industries Ltd.) was used to determine the accumulated triglycerides in the cell samples, as described previously[41,42].

Statistical Analysis

The data are presented as the mean ± standard deviation (SD). Each experiment was repeated three times ($n=3$). The statistical significance was determined using the unpaired Student's *t*-test for comparisons between the adipogenic induction groups and the control groups. A value of $p<0.05$ was considered to be significant.

RESULTS

Spheroid Formation of Human ASCs in the TASCL Device

Human ASCs at two different densities (6.4×10^4 and 1.3×10^5 cells/device) were inoculated into the TASCL device and cultured for 2 days. Cell spheroids approximately 70 μm in diameter were observed in the microwells using a phase-contrast microscope (Fig. 1A, b). Human ASCs in each microwell formed a single spheroid aggregation about 50–200 μm in diameter. In the 2D monolayer culture, human ASCs at densities of 1.3×10^5 cells inoculated onto the 12-well plates and cultured for 2 days formed human fibroblast-like cells (Fig. 1B, e).

Induction of Adipogenic Differentiation of Human ASC Spheroids in the TASCL Device

After 2 days of culture in maintenance medium, human ASC spheroids were cultured on the TASCL device for an additional 3 days in adipogenic differentiation medium (Fig. 1A). We observed human ASC spheroids in both the maintenance medium and the adipogenic differentiation medium after 5 days of culture. The morphology of the 3D spheroids was unchanged before and after culture in the adipogenic differentiation medium, continuing to exhibit spherical aggregates (Figs. 1A, b, and 3). Furthermore, no marked differences in the morphology of the human ASC spheroids were noted between cells cultured in the maintenance medium and in the adipocyte differentiation medium under a phase-contrast microscope (Fig. 3C, E, G, and I vs. Fig. 3D, F, H, and J). The size of the 3D spheroids increased both with increasing initial cell seeding density (Fig. 3G and H vs. Fig. 3I and J).

In the 2D monolayer culture, we observed the morphology of human ASCs cultured in maintenance medium and in adipogenic differentiation medium using an optical microscope. After 5 days of culture in adipogenic differentiation medium, the cells showed round morphology with the accumulation of lipid droplets (Fig. 3B). In contrast, the morphology of the human ASCs in maintenance medium did not change after 5 days of culture (Fig. 3A).

Evaluation of the Adipogenic-Differentiated Human ASC Spheroids in the TASCL Device

The degree of adipogenic differentiation of human ASC spheroids was evaluated by Oil red O staining of lipid droplets (Fig. 4) and by analyzing the accumulation of triglycerides (Fig. 5). After 1 h of Oil red O staining, positively stained cells were observed in both the maintenance medium and adipogenic differentiation medium (Fig. 4E and F). In particular, intracellular lipid droplets were positively stained with Oil red O for human ASC spheroids cultured in adipogenic differentiation medium for an hour and stained more intensely after 24 h of staining (Fig. 4G and H). In addition, the intracellular lipid droplets were positively stained with Oil red O for human ASC spheroids cultured in maintenance medium for 24 h and stained more intensely as the initial cell seeding density decreased (Fig. 4G and I).

In the 2D monolayer culture, the adipogenic differentiation of the human ASCs in the adipogenic differentiation medium was confirmed by positive Oil red O staining (Fig. 4B and D). The differentiated cells had a round morphology with lipid droplets (Fig. 4D). In contrast, the human ASCs cultured in the maintenance medium were negatively stained and formed human fibroblast-like cells (Fig. 4A and C).

We quantitatively assayed the accumulated triglycerides in the differentiated cells as described previously[41,42]. Briefly, human ASCs seeded at a density of 1.3×10^5 cells were incubated in maintenance medium for 2 days and then further cultured in either maintenance medium (uninduced) or adipogenic induction medium (induced) for 3 days in a 12-well plate for 2D culture (Fig. 5A) or in the TASCL device for 3D culture (Fig. 5B). In the 2D culture, triglyceride accumulation was observed only for human ASCs cultured in adipogenic induction medium (Fig. 5A). In the 3D culture, triglyceride accumulation in human ASCs cultured in adipogenic induction medium was approximately twofold higher than that in cells cultured in maintenance medium (Fig. 5B).

DISCUSSION

In this study, we evaluated the adipogenic differentiation of human ASC spheroids in 3D culture. We created an in vitro microenvironment using the combinatorial TASCL device and attempted to differentiate the human ASCs. We observed greater adipogenic differentiation in the 3D cultures than in the 2D cultures. Culturing

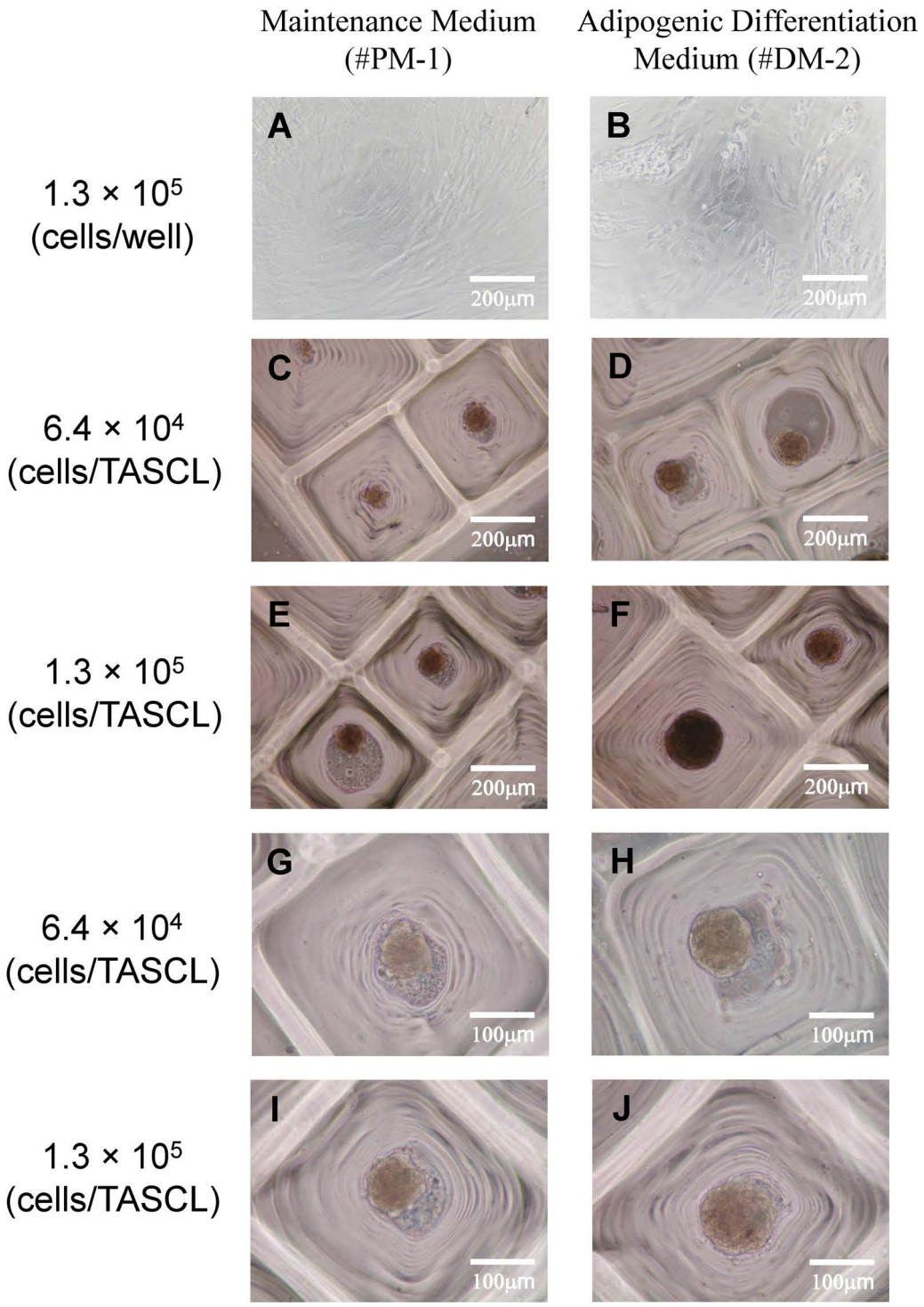

Figure 3. The adipogenic differentiation of human ASCs in 2D and 3D cultures. The morphology of adipogenic-differentiated human ASCs was observed using a phase-contrast microscope. In 2D culture, human ASCs at a density of 1.3×10^5 cells/well (A, B) were seeded onto a 12-well plate in maintenance medium and cultured for 2 days, and then further cultured in either maintenance medium (A) or adipogenic differentiation induction medium (B) for 3 days. In 3D culture, human ASCs were seeded onto the TASCL device at a density of 6.4×10^4 and 1.3×10^5 cells/device and cultured in maintenance medium for 2 days, and then further cultured in either maintenance medium (C, E, G, I) or adipogenic differentiation induction medium (D, F, H, J) for 3 days. Scale bars: 200 μm (A–F), 100 μm (G–J).

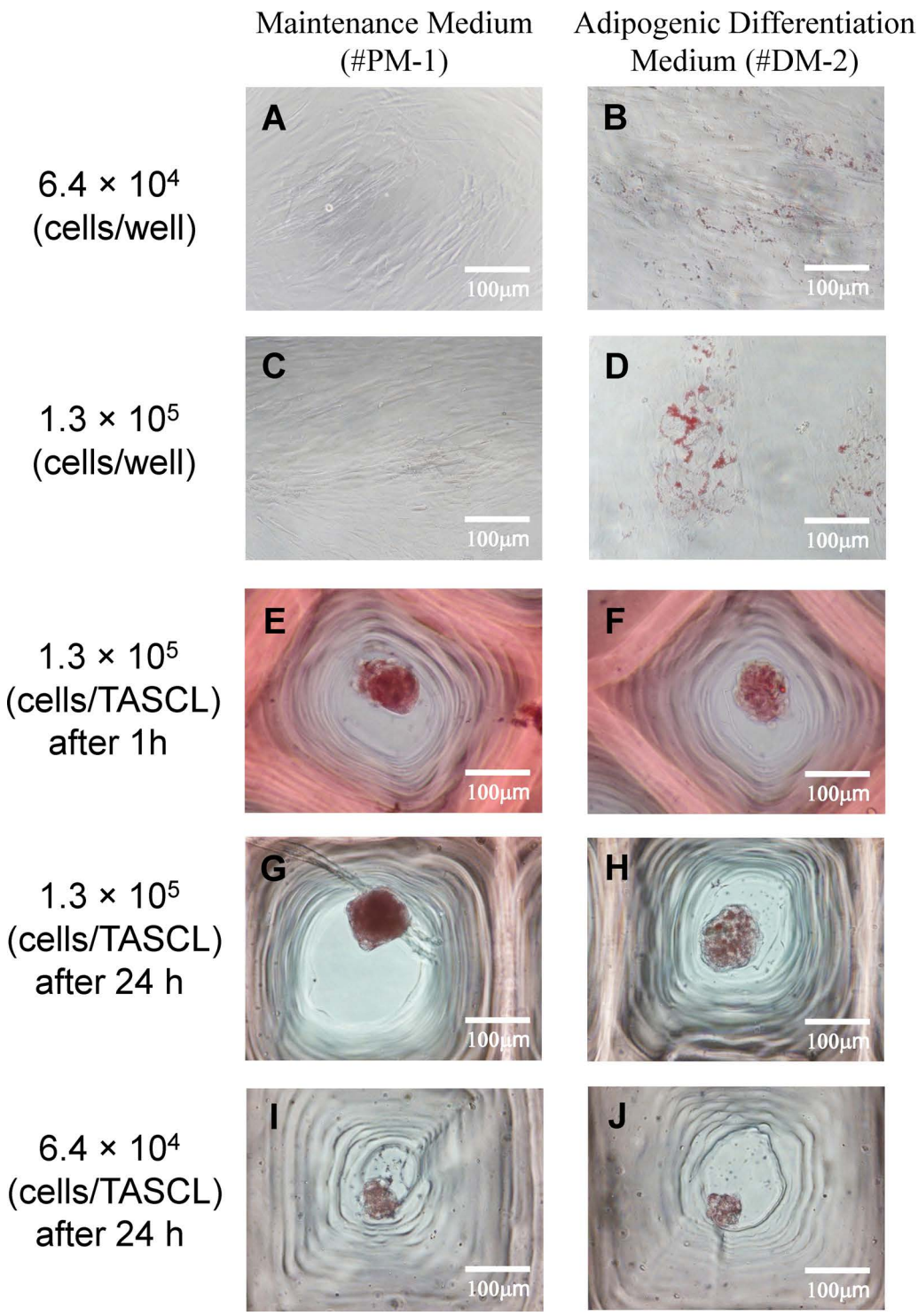

Figure 4. Phase-contrast images of Oil red O staining after adipogenic differentiation induction of human ASCs in 2D and 3D cultures. In 2D culture, human ASCs at a density of 6.4×10^4 and 1.3×10^5 cells/well were seeded onto a 12-well plate and cultured in maintenance medium for 2 days and then further cultured in either maintenance medium (A, C) or adipogenic differentiation induction medium (B, D) for 3 days. In 3D culture, human ASCs were seeded onto the TASCL device at a density of 6.4×10^4 (I, J) and 1.3×10^5 cells/device (E–H) and then cultured in either or both maintenance medium or adipogenic induction medium. The intracellular lipid droplets were stained with Oil red O after 1 h (A–F) and 24 h (G–J). Scale bars: 100 μm.

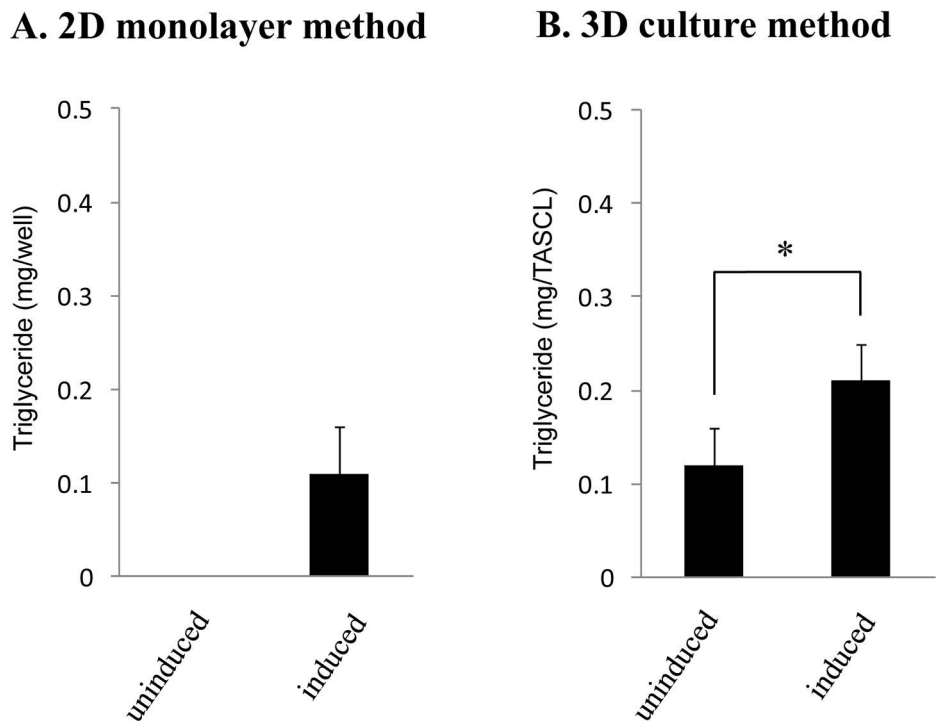

Figure 5. A quantitative analysis of the accumulated triglycerides after adipogenic differentiation of human ASCs in 2D and 3D cultures. Human ASCs seeded at a density of 1.3×10^5 cells/well (or device) were incubated in maintenance medium for 2 days and then further cultured in maintenance medium (uninduced) or adipogenic induction medium (induced) for 3 days in the 12-well plate (2D culture) (A) and in the combinatorial TASCL device (3D culture) (B). The amount of triglycerides accumulated in the cells was assayed using the Triglyceride E-test™ assay as described previously.

in adipocyte differentiation medium using the TASCL device promoted greater differentiation of human ASCs into adipogenic lineages than culturing in maintenance medium. These results indicated that the TASCL device is effective for creating in vitro microenvironments.

The 3D spheroids of human ASCs perfectly formed spherical aggregations in the TASCL device after 2 days of culture. Given that the size of each microwell in the TASCL device is different, the single cluster sizes of human ASCs varied (about 50–200 μm in diameter) (Figs. 3C–J and 4E–J). The TASCL device was coated with a hydrophilic layer, and an ultralow cell attachment surface on the bottom of each microwell in the TASCL device prevented cell adhesion. By creating a microenvironment in which the cells had difficulty adhering to the TASCL device itself, the cell–cell interactions of the human ASCs favored spherical aggregation. The cell scaffold (extracellular matrix as collagen, synthetic polymers as polystyrene, etc.) of a fabricated device for creating a microenvironment is extremely important[4,5,24–30,33,37–39]. We previously reported that type I collagen-coated plates and polystyrene plates used the same style of well bottom as the TASCL device[33]. However, the TASCL device is additionally treated with a hydrophilic layer. The cell attachment to cell scaffolds differs by cell type and culture conditions, and the resultant cell constructs can form spheroids, monolayers, and mixtures of both[33].

Human ASCs were cultured in the TASCL device for 5 days (Fig. 3) and then evaluated by Oil red O staining of lipid droplets (Fig. 4). The human ASC spheroids formed spherical aggregations, with no marked differences in shape noted between the maintenance medium and adipocyte differentiation medium. However, the intracellular lipid droplets were more strongly stained for human ASC spheroids (at 1.3×10^5 cells/TASCL) cultured in adipocyte differentiation medium (Fig. 4F and H) than for those cultured in maintenance medium (Fig. 4E and G).

The intracellular lipid droplets were positively stained with Oil red O for human ASC spheroids cultured in maintenance medium for 1 h (Fig. 4E) and 24 h (Fig. 4G). While the peripheral portion of the clusters promotes differentiation, the central portion of the clusters might not (Fig. 4G). We therefore performed the same staining experiment as above but changed the size of the human ASC spheroids (Fig. 4I and J). After 24 h of Oil red O staining, adipogenic differentiation of the human ASC spheroids was demonstrated by positive staining with

both the maintenance medium and the adipocyte differentiation medium (Fig. 4I and J). In the 2D monolayer culture, the adipogenic differentiation of human ASCs cultured for 5 days was demonstrated by positive Oil red O staining with adipocyte differentiation medium (Fig. 4B and D). As the culture progressed (7–10 days), the intracellular lipid droplets were positively stained with Oil red O more intensely.

We also confirmed the adipogenic differentiation of human ASCs by measuring the levels of triglycerides. The human ASCs in the 3D culture had greater triglyceride accumulation than those in the 2D culture. Furthermore, in the short culture period, the adipogenic differentiation proceeded more efficiently in the 3D culture than in the 2D culture. Although the culture medium in 3D culture is important for the efficient induction of differentiation, we also successfully induced adipogenic differentiation using only the TASCL device. These results indicate that the TASCL device is effective in creating an in vitro microenvironment.

In summary, a TASCL device effectively created an in vitro microenvironment and may be a useful tool for the induction of differentiation in 3D culture. The TASCL device functioned in the same way as other cell scaffolds for the adipogenic differentiation of human ASCs. The resultant human ASC spheroids were "adipose-like microtissues" that perfectly formed spherical aggregations and are expected to prove useful in regenerative medicine and cell transplantation.

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