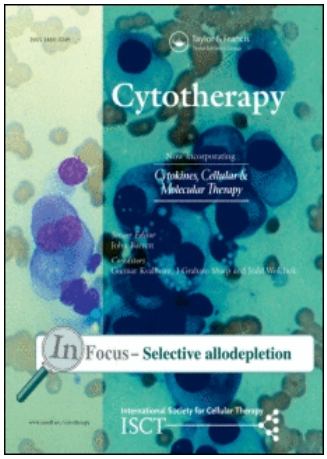

Cytotherapy
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713656803

Rapid expansion of human adipose-derived stromal cells preserving multipotency

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Online Publication Date: 01 January 2007
To cite this Article: Suga, H., Shigeura, T., Matsumoto, D., Inoue, K., Kato, H., Aoi, N., Murase, S., Sato, K., Gonda, K., Koshima, I. and Yoshimura, K. (2007) 'Rapid expansion of human adipose-derived stromal cells preserving multipotency', Cytotherapy, 9:8, 738 - 745
To link to this article: DOI: 10.1080/14653240701679873
URL: http://dx.doi.org/10.1080/14653240701679873

International Society for Cellular Therapy ISCT

Cytotherapy (2007) Vol. 9, No. 8, 738–745

informa healthcare

Rapid expansion of human adipose-derived stromal cells preserving multipotency

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Background

Adipose-derived stromal (stem) cells (ASC) have been shown to be of great therapeutic use in pre-clinical studies in diverse fields, but a standard expansion method has not been established. We investigated the effects of an endothelial growth medium (EGM-2) on ASC, focusing on proliferation and differentiation potentials.

Methods

ASC were cultured in EGM-2 and DMEM. Doubling time and total cell number were compared between the two media. The proliferative effect of each growth factor supplemented in EGM-2 was also examined. Cultured cells in each medium were examined for surface marker expression using flow cytometry. Differentiation into the adipogenic, chondrogenic and osteogenic lineages was analyzed after culture in each medium.

Results

ASC cultured with EGM-2 proliferated much more rapidly (10^5 times in 2 weeks) and reached the stationary phase earlier than those cultured with DMEM. Among the supplements contained in EGM-2, only fibroblast growth factor-2 (FGF-2) significantly promoted proliferation of ASC, although the proliferative effect of FGF-2 was much less than that of EGM-2, suggesting a synergism among other supplement factors. Flow cytometry and differentiation assays suggested that ASC cultured in EGM-2 preserved immunophenotype and differentiation capacity for at least three mesenchymal lineages (adipogenic, chondrogenic and osteogenic), similar to those cultured with DMEM.

Discussion

The present expansion method markedly accelerates proliferation of ASC, preserving their multipotent differentiation capacities, and lays the groundwork for establishing a practical route to mega-expansion of ASC for clinical applications.

Keywords

adipose-derived stem cells, differentiation, endothelial growth medium, fibroblast growth factor-2, mesenchymal stromal cells, multipotency, pre-adipocytes, proliferation, vascular endothelial growth factor.

Introduction

Human adherent stromal cells isolated from adipose tissue have been shown to have multipotency [1,2], able to differentiate not only into mesenchymal lineages, including endothelial cells [3–6] and cardiomyocytes [7–9] but also into neural cells [2] and hepatocytes [10]. These cells have been referred to by various names, including pre-adipocytes, vascular stromal cells, adipose-derived mesenchymal progenitor cells and adipose stromal cells. In this study, we refer to the cells as adipose-derived stromal (stem) cells (ASC). The characteristics of ASC have been studied extensively [11–15], as well as the potential clinical applications of ASC [3–7,16]. In addition, clinical trials have already begun involving enhancement of bone and adipose regeneration and angiogenesis [17–20].

Adipose tissue is thought to be a promising source of multipotent stromal cells because it can be harvested in relatively large quantities (100 mL–> 1 L) using liposuction, with minimal morbidity. Although ASC may be used clinically without cell expansion, because of the large

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DOI: 10.1080/14653240701679873

quantities, it is of great value to culture and expand ASC safely and effectively, without losing their multipotency, for manipulation and further development of cell-based therapies. There have been some reports indicating enhanced proliferation of human ASC using specific culture media with supplements. It has been shown that fibroblast growth factor-2 (FGF-2) is released by ASC, enhances proliferation [21–24] and maintains the adipogenic potential of ASC [21]. It has been suggested that FGF-1 and epidermal growth factor (EGF) act as stimulators of both ASC proliferation and differentiation [25–27]. Platelet-derived growth factor (PDGF) [25,28], tumor necrosis factor-α (TNF-α) [29] and insulin-like growth factor-1 (IGF-1) [29] have also been shown to promote ASC proliferation, and the former two factors were suggested to have inhibiting effects on ASC differentiation [25,29]. However, it has not been shown whether ASC expanded by these methods preserves their multipotency or not.

In this study, we investigated the effects of an endothelial growth medium (EGM-2®; Cambrex, Walkersville, MD, USA) on culturing human ASC, focusing on proliferation and differentiation potentials, and found that it markedly expands ASC, preserving multipotency. EGM-2 is usually used to support the growth of endothelial cells. In recent studies, EGM-2 has been used for culture of non-endothelial cells [16,30,31]. ASC have usually been cultured in DMEM or DMEM/F12 medium, and the effects of EGM-2 on ASC have not been reported.

Methods

Cell isolation and culture

We obtained liposuction aspirates from 12 healthy female donors undergoing liposuction of the abdomen or thighs, after informed consent using an IRB-approved protocol. The stromal vascular fraction containing ASC was isolated from the fatty portion of liposuction aspirates, as described previously [15]. Briefly, the aspirated fat was washed with PBS and digested on a shaker at 37°C in PBS containing 0.075% collagenase for 30 min. Mature adipocytes and connective tissues were separated from pellets by centrifugation (800 *g*, 10 min). The cell pellets were resuspended, filtered with a 100-μm mesh (Millipore, Billerica, MA, USA), plated at a density of 5×10^5 nucleated cells/100-mm dish, and cultured at 37°C in an atmosphere of 5% CO_2 in humid air. The culture medium was (1) DMEM (Nissui Pharmaceutical, Tokyo, Japan) containing 10% FBS or (2) EGM-2 containing 2% FBS. Endothelial basal medium (EBM; Cambrex) is the basal medium for EGM-2. EGM-2 does not contain any animal-derived factors but does contain FGF-2, vascular endothelial growth factor (VEGF), IGF-1, EGF, ascorbic acid, hydrocortisone, GA-1000 (gentamicin and amphotericin-B) and heparin, although the concentration of each agent is not disclosed. Freshly isolated cells were cultured for 7 days and the first culture was defined as passage 0. The medium was replaced every 3 days. Cells were passaged every week by trypsinization.

Measurement of doubling time and total cell number

During cell culture in each medium, the doubling time was measured at passages 0, 1, 2 and 3 by seeding ASC at a density of 1×10^5 cells/10-cm dish. After cells reached the logarithmic growth phase, they were sequentially trypsinized every 48 h and counted with a cell counter (NucleoCounter; Chemometec, Allerod, Denmark). The doubling time was calculated according to the following formula: doubling time $= 48\ \text{h}/\log_2(N_2/N_1)$, where N_1 is the first cell count and N_2 is the cell count 48 h later. The total cell number after the initiation of culture in each medium was also measured by seeding ASC (passage 0) at a density of 1×10^4 cells/3.5-cm dish and culturing the cells until they reached the stationary phase.

Measurement of the proliferative effect of supplemented growth factors

To examine the proliferative effect of each growth factor supplemented in EGM-2, ASC were cultured in medium supplemented with a single growth factor (VEGF, EGF, IGF-1 or FGF-2). EBM containing 2% FBS was used as the control medium. ASC (passage 0) were seeded at a density of 1×10^4 cells/well in a 6-well plate. Cells were cultured in the control medium (2% FBS), supplemented medium (0.1, 1 or 10 ng/mL of each growth factor) (2% FBS), DMEM (10% FBS) or EGM-2 (2% FBS). The number of cells after 7 days of culture was counted using a cell counter.

Flow cytometry of cultured cells

Cultured cells in each medium were examined for surface marker expression using flow cytometry. The following MAb conjugated to fluorochromes were used: anti-CD29–PE, CD31–PE, CD34–PE, CD45–PE, CD90–PE, CD146–

PE (BD Biosciences, San Diego, CA, USA), CD105–PE (Serotec, Oxford, UK) and Flk-1-PE (Techne, Minneapolis, MN, USA). Control MAb were included for all fluorochromes. Cells were incubated with directly conjugated MAb for 30 min, then washed and fixed in 1% paraformaldehyde. Cells were analyzed using an LSR II (Becton Dickinson, San Jose, CA, USA) flow cytometry system. Data acquisition and analysis were then performed (Cell Quest software; Becton Dickinson). Gates were set based on staining with combinations of relevant and irrelevant MAb so that no more than 0.1% of cells were positive using irrelevant Ab.

Induced differentiation of cultured cells

After culture in each medium for 2 weeks, differentiation into the adipogenic, chondrogenic and osteogenic lineages was examined. For adipogenic differentiation, cells were incubated for 4 weeks in DMEM containing 10% FBS supplemented with 0.5 mm isobutyl-methylxanthine (Sigma, St Louis, MO, USA), 1 μm dexamethasone, 10 μm insulin (Sigma) and 200 μm indomethacin. Adipogenic differentiation was visualized with Oil Red O staining. For quantitative analysis of lipid droplets, we measured Nile Red fluorescence using AdipoRed™ (Cambrex), with excitation at 485 nm and emission at 535 nm.

For chondrogenic differentiation, cells were incubated for 4 weeks in DMEM containing 1% FBS supplemented with 6.25 μg/mL insulin, 10 ng/mL TGF-β1 and 50 nm ascorbate-2-phosphate. Chondrogenic differentiation was visualized with Alcian Blue staining. For quantitative analysis, a micromass culture system was used, as reported previously [32]. Cells were plated in a 15-mL tube and cultured in the chondrogenic medium for 3 weeks. Then the diameter of a micromass was measured.

For osteogenic differentiation, cells were incubated for 4 weeks in DMEM containing 10% FBS supplemented with 0.1 μm dexamethasone, 50 μm ascorbate-2-phosphate and 10 mm β-glycerophosphate (Nacalai Tesque, Kyoto, Japan). Osteogenic differentiation was visualized with von Kossa staining. For quantitative analysis of total calcium, calcium deposition was evaluated based on the ortho-cresolphthalein complexone (OCPC) method with the Calcium C-Test Wako Kit (Wako Chemicals, Osaka, Japan) according to the manufacturer's instructions.

Statistical analyzes

Results are expressed as mean ± SEM. Welch's *t*-test was used to compare each parameter. A value of $P < 0.05$ was considered significant.

Results

Doubling time and total cell number

The doubling time of ASC cultured with EGM-2 was significantly shorter than that of cells cultured with DMEM at each passage (19.3 ± 2.1 h vs. 39.8 ± 6.8 h at passage 0; 15.6 ± 1.1 h vs. 55.1 ± 3.5 h at passage 1; 20.3 ± 0.7 h vs. 52.0 ± 2.4 h at passage 2; 26.5 ± 1.1 h vs. 54.3 ± 6.4 h at passage 3; Figure 1A). The total cell number showed that ASC cultured with EGM-2 proliferated more rapidly and reached the stationary phase earlier than those cultured

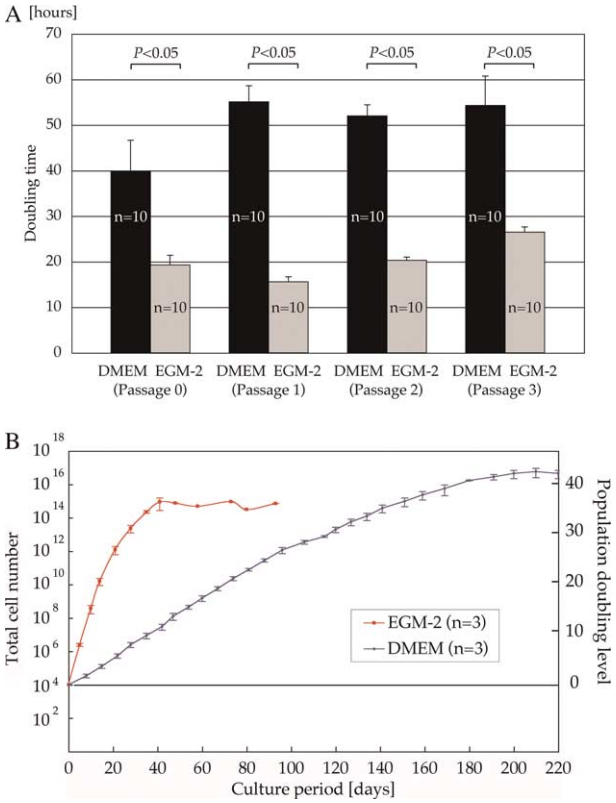

Figure 1. *(A) Doubling time at passages 0–3. The doubling time of ASC cultured with EGM-2 was significantly shorter than for those cultured with DMEM at each passage. (B) Total cell number and population doubling level after the initiation of culture with DMEM or EGM-2. ASC cultured in EGM-2 proliferated more rapidly and reached the stationary phase earlier than those cultured in DMEM. After 40 days of culture with EGM-2, the result was obtained from one sample.*

with DMEM (40 days vs. 200 days), although the maximum population doubling level of ASC was similar when cultured with EGM-2 or DMEM (35–40 with EGM-2 vs. 40–45 with DMEM; Figure 1B). Differentiation assays were performed using ASC cultured with each medium for 2 weeks and, at that stage, ASC cultured with EGM-2 supposed to be 10^5 times (10^{10} vs. 10^5) compared with those cultured with DMEM (Figure 1B).

Proliferative effect of each growth factor

At the concentrations tested, VEGF, EGF and IGF-1 showed no significant proliferative effect on ASC cultured in EBM containing 2% FBS. FGF-2, at a density of 0.1, 1 or 10 ng/mL, significantly promoted proliferation of ASC compared with the control. However, the proliferative effect of FGF-2 was much less than that of EGM-2 containing all of the growth factors, indicating a synergistic effect of supplemented growth factors (Figure 2).

Flow cytometry

Flow cytometry of ASC cultured in DMEM and EGM-2 showed no significant differences, except for CD105 at passages 1, 2 and 3 (Table 1). Both cell populations uniformly expressed mesenchymal markers (CD29 and CD90) and were devoid of hematopoietic cell marker CD45. Expressions of CD34 (stem cell marker), CD31 (endothelial cell marker), CD146 (endothelial cell and vascular mural cell marker) and Flk-1 (VEGFR-2) were similar in both cell populations, and CD34 expression of ASC markedly decreased at passage 1 in both media.

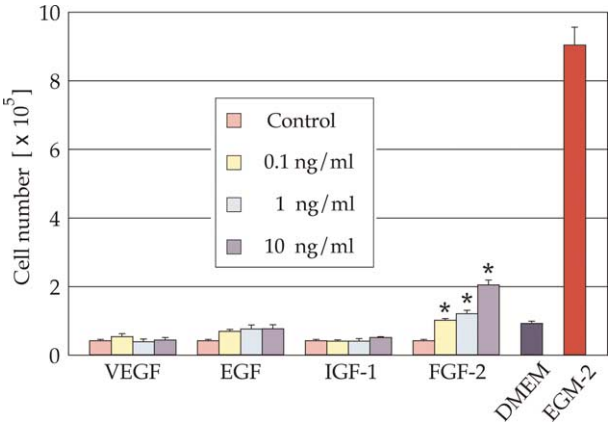

Figure 2. *Cell number after 7 days of culture in EBM (2% FBS) supplemented with one of the following growth factors: VEGF, EGF, IGF-1 or FGF-2* (n = *3). FGF-2, at a density of 0.1, 1 or 10 ng/mL, significantly promoted proliferation of ASC compared with control medium. The numbers of cells cultured in DMEM (10% FBS) or EGM-2 (2% FBS) are also indicated.* *$P < 0.05$.

Differentiation capacity

Both cell populations cultured in DMEM and EGM-2 for 2 weeks had similar capacities for differentiating into adipogenic, chondrogenic and osteogenic lineages. No morphologic differences between the two cell populations were observed during and after differentiation (Figure 3A). Quantitative analyzes (lipid droplets in adipogenic differentiation, micromass diameter in chondrogenic differentiation and total calcium content in osteogenic differentiation) also showed no significant differences between the two cell populations (Figure 3B).

Discussion

In this study, EGM-2 expanded ASC very rapidly while preserving their multipotency for at least 2 weeks; the proliferative efficiency of EGM-2 was 10^5 times that of DMEM in the first 2 weeks. A doubling time of ASC shorter than that shown in this study (15–20 h) has not been reported previously in the literature. EGM-2 contains 2% FBS and various growth factors, including FGF-2, VEGF, IGF-1 and EGF. The highly boosting effects of EGM-2 on ASC proliferation are suggested to result from the supplemented growth factors and other unknown synergistic effects, as discussed below. ASC cultured with EGM-2 proliferated much more rapidly and reached the stationary phase earlier than those cultured with DMEM (40 days vs. 200 days), although the maximum population doubling levels were similar between the two culture media (Figure 1B). The results suggest that the majority of ASC may have a limited capacity for self-renewal.

Serum concentrations can affect the proliferation activity of ASC. We have reported previously that the doubling time of ASC cultured with 15% FBS was significantly shorter than that of cells cultured with 10% FBS, although the culture media was M199 supplemented with FGF-1 in that study [15]. FBS is made by coagulations of fetal bovine whole blood; thus it is supposed to contain not only IGF-1, which is regularly present in serum, including platelet-poor plasma-derived serum, but also platelet-derived cytokines, such as PDGF and EGF [33]. In the present study, however, the doubling time of ASC cultured in DMEM was significantly longer than that for ASC cultured in EGM-2, in spite of the higher FBS concentration (10%) in DMEM compared with EGM-2 (2%). This

Table 1. Flow cytometry analyzes of cell surface marker Ag. Expression of mesenchymal markers (CD29, CD90, CD105), endothelial markers (CD31, CD146, Flk-1), a stem cell marker (CD34) and a hematopoietic marker (CD45) of ASC cultured with DMEM or EGM-2 was quantitatively examined at passages 0–3

	Percentage of positive cells for each surface marker			
	Passage 0	Passage 1	Passage 2	Passage 3
CD29				
DMEM	98.4 ± 0.1	98.8 ± 0.4	99.1 ± 0.2	99.4 ± 0.2
EGM-2	99.4 ± 0.0	99.3 ± 0.1	99.5 ± 0.1	94.5 ± 1.5
CD31				
DMEM	0.8 ± 0.3	0.4 ± 0.1	0.3 ± 0.2	0.1 ± 0.1
EGM-2	0.3 ± 0.2	0.5 ± 0.2	0.2 ± 0.0	0.2 ± 0.1
CD34				
DMEM	60.0 ± 16.5	4.1 ± 1.5	0.4 ± 0.1	1.2 ± 0.3
EGM-2	57.9 ± 16.6	0.2 ± 0.1	0.0 ± 0.0	0.2 ± 0.1
CD45				
DMEM	2.3 ± 0.3	0.7 ± 0.4	0.3 ± 0.1	0.6 ± 0.4
EGM-2	0.9 ± 0.1	0.7 ± 0.6	0.1 ± 0.0	0.4 ± 0.2
CD90				
DMEM	98.8 ± 0.5	99.7 ± 0.1	99.7 ± 0.1	99.7 ± 0.0
EGM-2	99.2 ± 0.3	99.2 ± 0.3	97.0 ± 0.1	81.8 ± 4.3
CD105				
DMEM	75.1 ± 8.5	92.3 ± 4.2	92.7 ± 1.4	93.2 ± 1.8
EGM-2	53.7 ± 18.9	37.2 ± 9.3	24.2 ± 16.3	22.9 ± 6.2
CD146				
DMEM	7.5 ± 3.8	25.4 ± 13.3	9.6 ± 3.5	15.9 ± 6.8
EGM-2	3.2 ± 2.2	7.4 ± 1.8	6.3 ± 2.4	2.2 ± 1.6
Flk-1				
DMEM	5.1 ± 0.9	3.9 ± 1.3	3.2 ± 1.4	2.0 ± 0.6
EGM-2	9.1 ± 4.6	7.8 ± 0.1	4.8 ± 3.2	1.1 ± 0.3

result suggests that the growth factors added to EGM-2 have greater effects on the proliferation activity of ASC than serum concentrations. In fact, a recent report has suggested that platelet-derived growth factors (not designated, but assumed to be PDGF and EGF) may reduce the proliferation activity and adipogenic differentiation capacity of ASC [23].

Among the growth factors contained in EGM-2, supplementation with IGF-1, EGF or VEGF (0.1, 1 or 10 ng/mL of each growth factor) did not significantly promote the proliferative activity of ASC in EBM cultures containing 2% FBS. The growth factors had some proliferative effects on ASC when added to serum-free media or SPPP, as reported previously [5,23,29]; however, any effects in this study using a low concentration of FBS with IGF-1 and EGF were subtle or masked. In this study, only FGF-2 showed a statistically significant promoting effect on ASC proliferation, which has been suggested in previous studies [21–24]. The results strongly suggest that FGF-2 is a critical growth factor for supplementation of serum-containing culture media. A previous study suggested that FGF-2 plays a critical role in self-renewal of ASC [21]. It has also been shown that FGF-2 added to SPPP increases the proliferation activity and adipogenic differentiation capacity [23]. Another study reported efficient proliferation of ASC transfected with the FGF-2 gene [34].

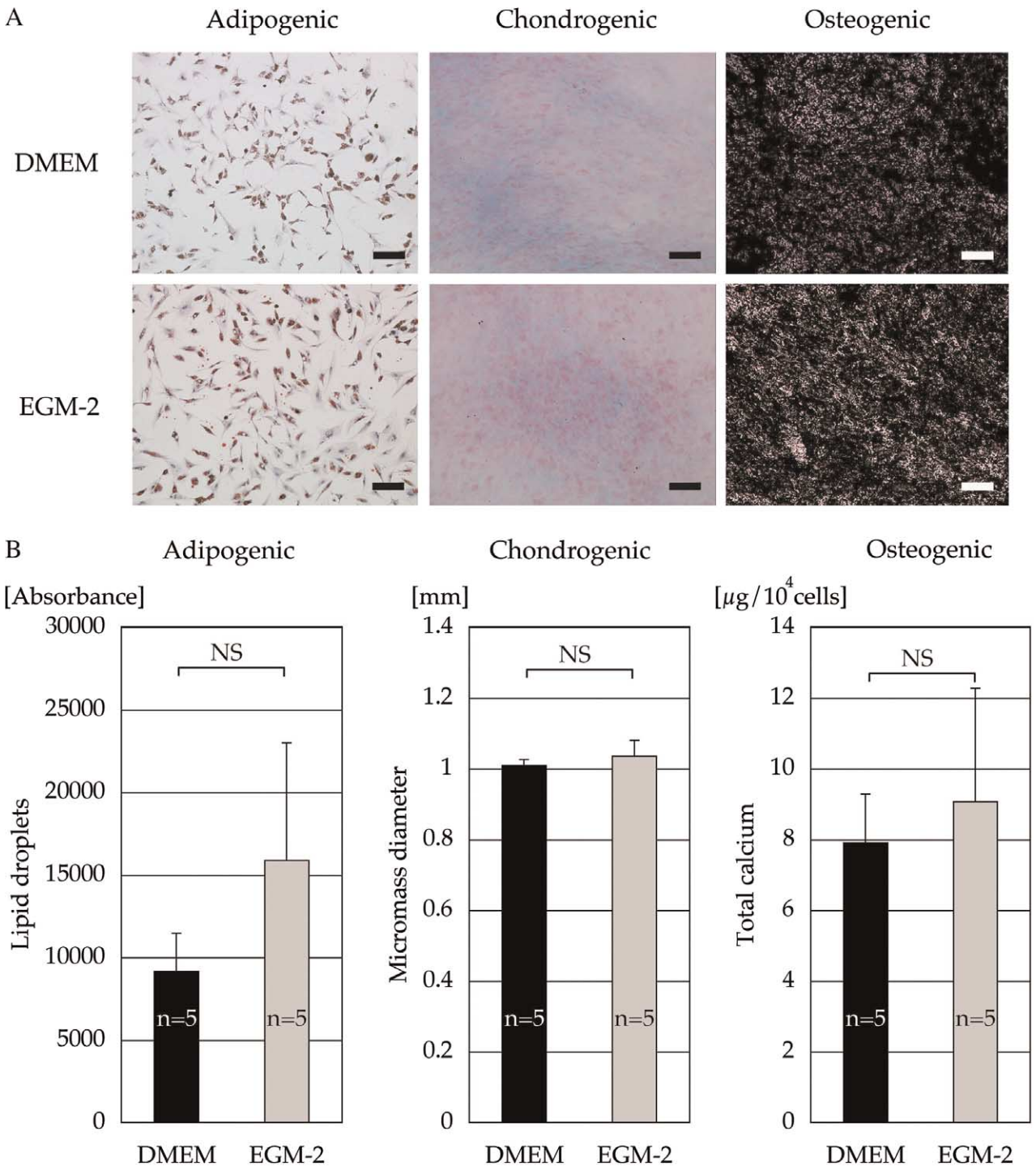

Figure 3. *(A) Microscopic results of cell differentiation. Both cell populations cultured in DMEM and EGM-2 for 2 weeks had similar capacities to differentiate into adipogenic, chondrogenic and osteogenic lineages. Adipogenic, chondrogenic and osteogenic differentiations were visualized with Oil Red O, Alcian Blue and von Kossa staining, respectively. Scale bar = 100 μm. (B) Quantitative analyzes of cell differentiation. Differentiation potentials were evaluated by lipid droplet contents (adipogenic), micromass diameter (chondrogenic) and total calcium contents (osteogenic). No statistical significances were observed (adipogenic* $P = 0.31$, *chondrogenic* $P = 0.68$, *osteogenic* $P = 0.55$). *NS, no significant difference.*

However, in 7 days, EGM-2 expanded ASC significantly and several-fold more compared with FGF-2-supplemented EBM (Figure 2) and thus the effect of EGM-2 on ASC proliferation cannot be explained solely based on the influence of FGF-2 alone. It is likely that synergistic effects of the various growth factors and other factors contributed to the exceptional efficiency.

We have previously reported surface marker expression of freshly isolated and cultured ASC [15], although M-199 was used in the study. In the present study, the character of ASC expanded with EGM-2 did not appear to change significantly. ASC cultured with EGM-2 preserved differentiation capacities similar to those with DMEM, into at least three mesenchymal lineages: adipogenic, chondrogenic and osteogenic. In addition, flow cytometry of both populations showed no significant differences, except for CD105, and presented no increase of differentiation markers, such as CD31, suggesting that ASC remain in an undifferentiated and proliferating state. These results suggest that EGM-2 accelerates expansion of ASC mainly by facilitating proliferation of undifferentiated cells. Although expression of CD105 has been used as one of the definitive markers of mesenchymal stromal cells, it may not strongly correlate with the multipotency.

Recent reports have shown that ASC can differentiate into endothelial cells *in vitro* under certain culture conditions using endothelial growth media and also *in vivo* [3–6]. ASC may be essentially common progenitors of adipocytes and vascular cells [5]. In most of the *in vitro* studies, a semi-solid medium, such as methylcellulose and Matrigel, was used, which may be key to endothelial differentiation of ASC [35]. Although EGM-2 containing VEGF was originally a medium for expanding endothelial cells, and ASC express Flk-1, a VEGF receptor, endothelial cell marker expression of ASC was not enhanced by EGM-2 in our study using cell culture on a plastic dish. In addition, hypoxic conditions have various influences on ASC [36–39], one of which is enhancing ASC secretion of angiogenic factors such as VEGF and HGF [36]. In the studies showing endothelial differentiation of ASC *in vivo*, ASC were transplanted to the ischemic hind limb or under other ischemic conditions [4–6], so a hypoxic condition may be an important factor in endothelial differentiation *in vivo*.

A number of pre-clinical studies with human ASC have been reported; in most, undifferentiated ASC were used, rather than ASC differentiated into a specific lineage, although the functional mechanism of transplanted ASC varied among studies. Transplanted ASC survive as undifferentiated cells and act as tissue-specific progenitors or provider cells of soluble factors in some studies [3,7,16]. In others, transplanted ASC differentiate into a specific lineage, such as bone and vessels, according to the circumstances of recipient sites [3,7,17]. For the therapeutic use of ASC, expansion of undifferentiated cells, rather than their differentiation into a specific lineage, is likely to be of great importance in the processing of the cells before transplantation. In clinical practice, a safer and more rapid expansion method is required in view of time and cost requirements. Although neither EBM nor EGM-2 is approved for clinical use at present, EGM-2 does not contain animal-derived factors and the FBS used in this study can easily be replaced with autologous serum or human allogenic serum. The present expansion method with EGM-2 has an exceptional efficiency and lays the groundwork for establishing a practical route to mega-expansion of ASC for clinical applications.

Acknowledgements

We are very grateful to Ayako Kurata, Akiko Matsuura and Satomi Kawarasaki for their technical assistance.
The authors declare that they have no competing financial interests.

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