Research Article

Expansion of Semi-Automatic Processed Human Adipose-Derived Stem Cells in Medium Supplemented with Autologous Serum and Antioxidants

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Abstract

Objective: Excised fat tissue (EFT) is an excellent source of adipose-derived stem cells (ADSCs) for autologous cell therapy. However, the current technique for excising fat tissues with conventional surgical scissors and using culture medium containing fetal bovine serum (FBS) may not comply with Good Tissue Practice (GTP) guidelines. We thus developed a semi-automated process complying with the abovementioned guideline to manufacture clinical-grade ADSCs from small volumes of EFT.

Methods: A semi-automated process was developed by using a disposable disperser tube operated on a Tube Disperser Workstation to obtain stromal vascular fraction (SVF) from EFT. A culture medium supplemented with human autologous serum (HAS) and mesenchymal stem cell culture adjuvant (MCA) consisted of basic fibroblast growth factor (bFGF/FGF-2) and antioxidants, was formulated to expand ADSCs from SVF in vitro.

Results: The semi-automated process can markedly enhance cell yields and reduce the operation time for mincing tissues from an hour down to a few minutes. The growth of ADSCs was slower in the 10% HAS medium than that in medium supplemented with FBS, but their growth in the 10% HAS medium containing MCA (10% HAS+MCA) was superior to both media. The 10% HAS+MCA medium also promoted adipogenesis, osteogenesis, chondrogenesis and increased the expression of CD44, CD73 and the stemness genes in the ADSCs.

Conclusion: Our novel semi-automated process provides an efficient isolation of SVF, and the 10% HAS+MCA medium accelerates the growth of ADSCs from small amounts of EFT without losing their differentiation ability.

Keywords: Disposable disperser tube; Adipose-derived stem cells; Antioxidants; Fibroblast growth factor-2; Autologous serum

Abbreviations: ADSCs: Adipose-derived Stem Cells; HAS: Human Autologous Serum; GTP: Good Tissue Practice; GMP: Good Manufacturing Practice; SVF: Stromal Vascular Fraction; MCA: Mesenchymal Stem Cell Culture Adjuvant; FBS: Fetal Bovine Serum; GVHD: Graft-Versus-Host Disease; AFT: Aspirated Fat Tissues; MSCs: Mesenchymal Stem Cells; BMI: Body Mass Index; EFT: Excised Fat Tissues; SFM: Serum-Free Medium; IRB: Institutional Review Board; IMDM: Iscove's Modified Dulbecco's Medium; NAC: N-acetyl-L-cysteine; AsA-2P: Ascorbic Acid-2-Phosphate; PI: Propidium Iodide; cbfa1: Core-Binding Factor Subunit Alpha-1; OC: Osteocalcin; COL IA1: Collagen Type I, Alpha 1; PPARγ: Proliferator-Activated Receptor Gamma; aP2: Adipocyte Fatty Acid-Binding Protein; ACAN: Aggrecan; COL IIA1: Collagen Type II, Alpha 1; ALP: Alkaline Phosphatase; SOX2: Sex-Determining Region Y-box 2; CXCR4: C-X-C Chemokine Receptor Type 4; TERT: Telomerase Reverse Transcriptase; FGF-2: Fibroblast Growth Factor-2; EGF: Epidermal Growth Factor; PDGF: Platelet-Derived Growth Factor; VEGF: Vascular Endothelial Growth Factor; HGF: Hepatocyte Growth Factor; IGFBP: Insulin-Like Growth Factor Binding Protein; IGF: Insulin-Like Growth Factor; PL: Platelet Lysate; p21: Cyclin-Dependent Kinase Inhibitor 1A; p27: Cyclin-Dependent Kinase Inhibitor 1B.

Introduction

Freshly isolated, non-expanded stromal vascular fraction (SVF) is considered safe and effective for cell therapy [1,2]. Adipose tissue is an easily accessible and rich source of SVF [3-6]. Recently, autologous SVF has been clinically tested for breast reconstruction and augmentation [7], facial lipoatrophy [8], breast implant complications [9], depressed scars [10], traumatic calvaria defects [11], Crohn's disease fistulae [12] and graft-versus-host disease (GVHD) [13]. The SVF from these studies were isolated from aspirated fat tissues (AFT) by liposuction [14]. SVF contains mesenchymal stem cells (MSCs), namely adipose-derived stem cells (ADSCs), which can differentiate into adipocytes,

chondrocytes, osteoblasts, endothelial cells and keratocytes for further applications in regenerative therapy and tissue engineering [2-4, 15]. However, only 1%–5% of cells from non-manipulated SVF will become ADSCs, which provides insufficient cell yield for patients with low body mass index (BMI).

ADSCs derived from the direct excision of orbital fat mass [3,16] and buccal fat pad excised by surgery [17] compared to those isolated from lipoaspirated abdominal subcutaneous fat have similar differentiation potential [18]. Although the size of excised fat tissue (EFT) samples are commonly smaller, ADSC yields from EFT are often greater than that from AFT [6], suggesting that EFT could be an alternative source of ADSCs for individuals with lower BMI [3,6,16,18]. Currently, EFT must be minced manually with surgical scissors in a biological safety cabinet prior to further incubation with collagenase for SVF isolation [3,18]. The manual method presents major limitations including a lengthier time frame required to process the tissue for the extraction of SVF and variability in the resulting isolated cells due to intra-operator processing. The repeated use of surgical scissors also presents risks of cross-contamination. Several bench-top closed systems have been developed for SVF isolation from AFT [1], including the Cytori Celution™ system [19] and the Tissue Genesis' TGI 1000™. However, an appropriate automated system for the isolation of SVF or ADSCs from EFT has yet to be developed.

Fetal bovine serum (FBS) are commonly used for the *ex vivo* expansion of MSCs, which is a potential source of zoonotic or other infectious agents such as prions, viruses and mycoplasma. FBS is a chemically ill-defined supplement with high batch-to-batch variability, which may lead to unpredictable cell growth or variation in differentiation [20-23]. On the other hand, the expansion of MSCs using human serum-supplemented medium is less efficient [24-25]. Several serum-free media (SFMs) have been recognized to be safe and free from animal products and related issues [26]. SFMs also do not affect the immunophenotype, proliferation potential and multipotency of ADSCs [22,27-33]. These SFMs include MesenCult®-XF Medium (Stemcell Technologies, Canada) [34], StemPro® MSC SFM (Life Technologies, US), MSC Nutristem® XF Medium (Biological Industries, Israel) and BD Mosaic™ (Becton Dickinson, US). However, the cost of these SFMs and their lack of clinical validation limit their clinical applications.

Autologous cell transplantation is the best choice for patients who require cell therapy, where EFT harbors an excellent source of autologous ADSCs. We developed a semi-automated process complying with Good Tissue Practice (GTP) and Good Manufacturing Practice (GMP) guidelines to replace the current manual techniques to minimize human-induced variability and comply with clinical trial standards. In addition, we have also formulated a human autologous serum (HAS)- and antioxidant-supplemented medium for the expansion of ADSCs, and compared it with media containing either animal sera or HAS alone.

Materials and Methods

Clean room

Procedures for the isolation of SVF and expansion of ADSCs from EFT were conducted in Biological Safety Cabinets (Model 1286, Thermo Scientific Forma Class II Type A2, Thermo Scientific, Waltham, MA) in a clean room (ISO14644 Class 7) located in Gene and Stem Cell Production Laboratory of Hualien Buddhist Tzu Chi General Hospital.

Each suite is supported by positive-pressure (10-15 Pa), HEPA-filtered air, at a temperature of 22°C and relative humidity of 55-65%. The flow suite is unidirectional, with entry and exit air locks. All personnel were trained in respect to the GMP guidelines.

SVF isolation from EFT

The experimental protocol was approved by the Institutional Review Board (IRB) of Min-Sheng General Hospital, Taoyuan, Taiwan. EFT from abdominal subcutaneous fat was obtained from 11 pregnant women (average age of 33.7 ± 3.3). About 25 grams of adipose tissues were collected from each donor, and the tissues were minced either by disposable disperser tubes (DT-20 gamma, IKA ULTRA TURRAX Tube drive, Staufen, Germany) or manually with surgical scissors. The process of isolation is shown in Figure 1. About 3 grams of EFT was resuspended in 12 mL of Iscove's modified Dulbecco's medium (IMDM; GIBCO-Invitrogen, Grand Island, NY), placed in a 15 mL disposable disperser tube, and homogenized by centrifugation at 1200 rpm for 10 seconds thrice. Collagenase (Animal Origin Free)-A (LS004154, Worthington Biochemical Corporation, Lakewood, NJ; final concentration: 0.4 mg/mL, volume: 15 mL) was added for enzymatic digestion, and incubated in MO-01 hybridization oven (15 rpm, 37°C; Double Eagle Enterprise, New Taipei City, Taiwan) for 8 hours (Figure 1D). After dispersing the digested adipose tissues several times with a 10 ml sterile pipette (CORNING, Tewksbury, MA), the digested tissues were centrifuged at 2000 rpm for 10 min to generate SVF pellets (Figure 1E and 1F). The SVF pellet was then resuspended in PBS and filtered through a 70 μm strainer (Becton Dickinson, San Jose, CA) and the SVF cells were ready for ADSCs culture.

ADSCs culture

HAS was collected from each respective donor in Serum/Plain Tube (Cat# 366430; Becton Dickinson) with informed consent. The SVF cells were propagated in MSC maintenance medium containing IMDM, 2 mM L-glutamine (GIBCO-Invitrogen), 2% or 10% HAS and MSC-culture adjuvant (MCA). The MCA is consisted of 10 ng/mL fibroblast growth factor 2 (FGF-2, R&D Systems, Minneapolis, MN), 2 mM N-acetyl-L-cysteine (NAC, Sigma, St. Louis, MO) and 0.2 mM L-ascorbic acid-2-phosphate (AsA2P, Sigma). Other media consisted of IMDM, 2 mM L-glutamine with 10% FBS (MSC-Qualified; GIBCO-Invitrogen) or 10% HAS, together with 10 ng/mL FGF-2 in all conditions, were used for comparison in the following assays (see below). The cells were cultured at 37°C in a humidified, 5% CO_2 incubator (Forma Series II Model 3110, Thermo Scientific). Cells at passages 1-3 of different donors were used in the following experiments.

Cell number and viability assays

For cell number and viability assays of SVF, cells were counted using an automatic cell counter, ADAM-MC (NanoEnTek, Seoul, Korea), with Propidium Iodide (PI) according to the manufacturer's instructions. For cell proliferation and viability assays, ADSCs were detached by trypsinization and then counted in triplicates using Vi-CELL AS cell counter (Beckman Coulter, Indianapolis, IN) as previously described [35]. For total ADSCs yield, the attached cells from SVF culture under various expansion media were detached by exposure to trypsin-EDTA on the 5th day.

Flow cytometric analysis

For the characterization of cell surface phenotype, the ADSCs were cultured in 75 cm² tissue culture flasks (Becton Dickinson) at an initial
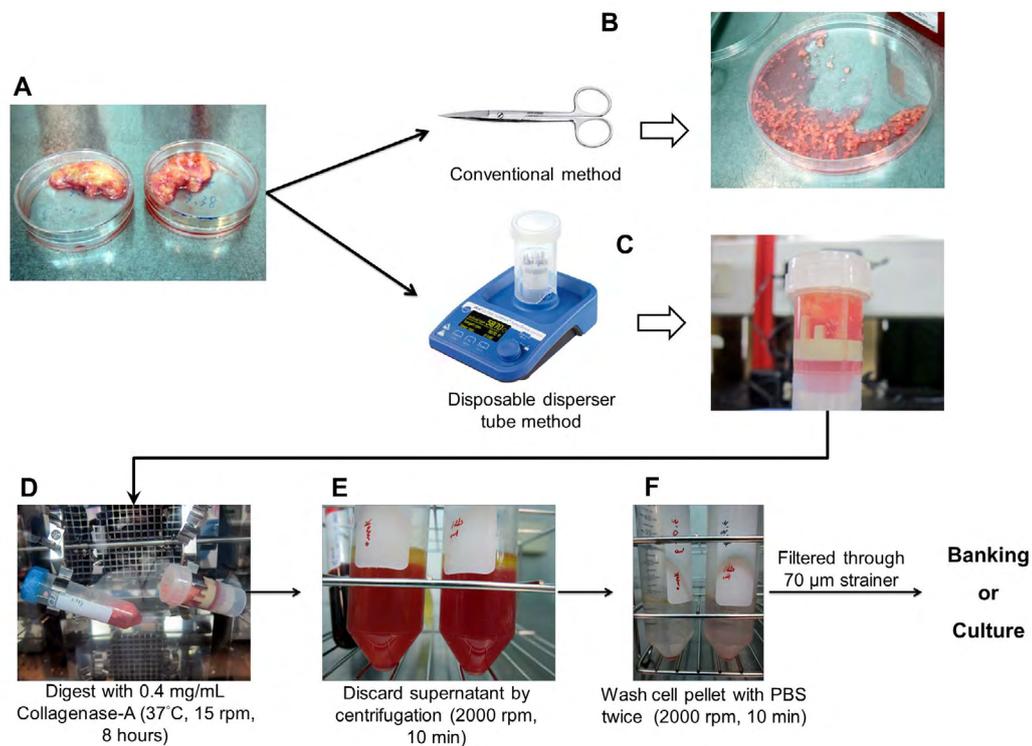
Figure 1: Overview of SVF isolation from fat tissues. In the following experiments, the fat tissues were all excised from abdominal subcutaneous tissues. (A) 3 g of the excised fat tissues were placed in the culture dishes and cut into small fragments either (B) by surgical scissors (conventional method) or (C) by disposable disperser tube method (our newly developed technique). (D) Samples were digested with collagenase in hybridization oven and (E-F) SVF pellets were generated by centrifugation.

density of 1000 cells/cm^2 in a humidified 5% CO_2 and 37°C incubator. After 6 days, single-cell suspensions were obtained by trypsinization, resuspended in culture medium, and further incubated at 37°C in CO_2 incubator for 30 minutes. Cells were stained with anti-human CD13, CD34, CD44, CD73, CD90 and CD105 (Becton Dickinson) conjugated with fluorescein isothiocyanate or R-phycoerythrin for 1 hour at 4°C (for negative controls, primary antibodies were omitted). The expression profiles were examined by flow cytometry (FACSCalibur, Becton Dickinson) and analyzed by CXP Software (Beckman Coulter, CA).

Analysis of gene expression and karyotyping

After incubation under different culture media for 6 days (n=3 for each medium, initial cell density of 1000 cells/cm^2), the ADSCs were harvested to examine the expression of stemness and growth factor genes. Isolation of total RNA, cDNA synthesis and quantitative polymerase chain reaction (qPCR) were carried out as previously described [36]. Briefly, total RNA was isolated from cells using the RNeasy kit (Qiagen, Valencia, CA), and cDNA was synthesized from 10 µg of total RNA using the Advantage RT-for-PCR kit (Clontech, Palo Alto, CA). qPCR and product detection were performed using the Fast Start Essential DNA Green Master (Roche, Indianapolis, IN) and the PikoReal Real-Time PCR System (Thermo Scientific) with the gene-specific primers as listed in Table 1. The results of amplification were analyzed using PikoReal 2.0 software (Thermo Scientific, PA). Gene expression levels were normalized to *β-actin*, which served as internal control.

For karyotyping analysis, 75 cm^2 tissue culture flasks seeded with ADSCs in 10% HAS+MCA-supplemented medium with an initial density of 3000 cells/cm^2 were placed in a CO_2 incubator. Once the cells reached 50–60% confluence, they were washed twice with phosphate buffered saline (PBS), detached by 0.25% trypsin-EDTA (Gibco BRL) and then replated at a density of 3000 cells/cm^2 under the same culture conditions. Karyotyping of ADSCs was performed following one month culture by established protocols [37].

In vitro differentiation

After incubation under various culture media conditions (10% FBS, 10% HAS, 10% HAS+MCA or 2% HAS+MCA, n=3 for each medium) for 6 days, the ADSCs were seeded into 6-well plates (Becton Dickinson) for differentiation assays. Osteogenesis, adipogenesis and chondrogenesis of ADSCs were induced according to established protocols [38,39]. Osteogenic differentiation was evaluated by alkaline phosphatase (ALP) staining and the expression of *cbfa1* (core-binding factor subunit alpha-1), *OC* (osteocalcin) and *COL IA1* (collagen type I, alpha 1) on day 14. Adipogenic differentiation of ADSCs was evaluated using Oil red O staining and the expression of *PPARγ* (proliferator-activated receptor γ) and *aP2* (adipocyte fatty acid-binding protein) on day 7. Chondrogenic differentiation was evaluated using Alcian Blue (Sigma) staining of sulfated proteoglycan-rich matrix (blue color) and the expression of *ACAN* (aggrecan) and *COL IIA1* (collagen type II, alpha 1) on day 14. Methods for ALP staining, Oil red O staining and Alcian Blue staining were as reported previously [4,33,35].

Gene	Primer sequences	Product size (bp)
β-actin	S: 5`- CGCCAACCGCGAGAAGAT-3` A: 5`- CGTCACCGGAGTCCATCA -3`	168
Nanog	S: 5`-AATACCTCAGCCTCCAGCAGAT-3` A: 5`-TGCGTCACACCATTGCTATTCTT-3`	148
SOX2	S: 5`- CCTCCGGGACATGATCAG-3` A: 5`- TTCTCCCCCCTCCAGTTC-3`	178
TERT	S: 5`- AGTGGATTCGCGGGCACAGA-3` A: 5`- TTCCCACGTGCGCAGCAGGA-3`	257
CXCR4	S: 5`-CGTGGAACGTTTTTCCTGTT-3` A: 5`-TGTAGGTGCTGAAATCAACCC-3`	129
IGF-1	S: 5`-AAGATGCACACCATGTCC-3` A: 5`-TGTTGAAATAAAAGCCCCTG-3`	157
HGF	S: 5`-TGTTCCCTTTTTTGGGTAAGC-3` A: 5`-CCCATTTGCCACAGAAAGTT-3`	148
EGF	S: 5`-CTAATCACCTACTCAATGCCTGG-3` A: 5`-TGATTCTCCCAGTACTCTTACTTGG-3`	109
OC	S: 5`-CAAAGTCTAACTAGGGATACC-3` A: 5`-AGAGATGAGTCTGTCCTG-3`	150
Cbfa1	S: 5`-TGGCAGCACGCTATTAAATC-3` A: 5`-TCTGCCGCTAGAATTCAAAA-3`	103
COL IA1	S: 5`- GACTCTAAGATCAGAGACGGAGAC-3` A: 5`- TCGCTGACATCTCCATTCATTCAC-3`	250
PPARγ	S: 5`-TTGCTGTCATTATTCTCAGTGGA-3` A: 5`-GAGGACTCAGGGTGGTTCAG-3`	124
aP2	S: 5`-CAATCTAGCAGACGGAACTGAA-3` A: 5`-CCGTTTGAATTTTCCAATAAGTTT-3`	76
ACAN	S: 5`-TACACTGGCGAGCACTGTAAC-3` A: 5`-CAGTGGCCCTGGTACTTGTT-3`	71
COL IIA1	S: 5`-GAATAGCACCATTGTGTAGGAC-3` A: 5`-AATGCCCCCTGAGTGAC-3`	97

S: Sense; A: Antisense

Table 1: Primers used for quantitative polymerase chain reaction.

Cytokine and growth factor array

Human cytokines and growth factors from culture supernatants were detected and quantitated using Human Cytokine Antibody Array 1 (Cat #AAH-GF-1, RayBiotech, Norcross, GA) according to the manufacturer's instructions. Cytokine and growth factor expression from culture supernatants were quantified by ImageJ 1.40 g software (NIH), using the positive control (POS) on the same membrane as the internal control. Data are presented as the relative ratio of cultured to un-cultured groups.

Statistical analysis

Statistical analysis for cell proliferation, gene expression and cytokine contents in supernatants were performed with Microsoft Excel using t-tests. Cell numbers and viabilities following extraction via the two differential methods were analyzed with Wilcoxon signed rank test using Prism 4 (GraphPad Software, CA). $p<0.05$ was considered statistically significant. Experiments were performed at least twice, and each data point is presented as mean ± SD.

Results

SVF isolation from EFT by disposable disperser tubes

We have developed a novel semi-automated method to isolate SVF from EFT. For comparison, the EFT was minced either with surgical scissors (Figure 1B) or by the semi-automated disposable disperser tubes (Figure 1C). The processed fat tissues were then digested with collagenase for 8 hours (Figure 1D). Finally, SVF cells were isolated from the digested fat tissues by centrifugation into cell pellets (Figure 1E and 1F). The semi-automated disposable tube tissue dispersion method reduced the operation time for mincing tissue from approximately one hour to a few minutes, and also avoided the use of scissors, a potential source of contaminants.

Cell yield and viability of SVF and ADSCs prepared using the semi-automated tube disperser and manual operation

The yield and viability of SVF and ADSCs prepared using either the semi-automated tube disperser method or the manual operation with surgical scissors was compared in 9 donors (Figure 2). Three grams of fat tissues were used for both methods. To compare total yield of ADSCs, SVF cells were seeded at the same initial cell density (10,000 cells/cm^2) and incubated in 10% HAS+MCA-supplemented medium for 5 days, and then detached using trypsin-EDTA. The cell yield of SVF prepared using tube disperser was 1.3-10.2 folds higher than the conventional method. The cell viability of SVF prepared using tube disperser was 0.9-2.4 folds higher than the conventional method (Figure 2A). Moreover, the total yield of ADSCs was 1.2-7.8 folds higher when compared to the conventional method, and the
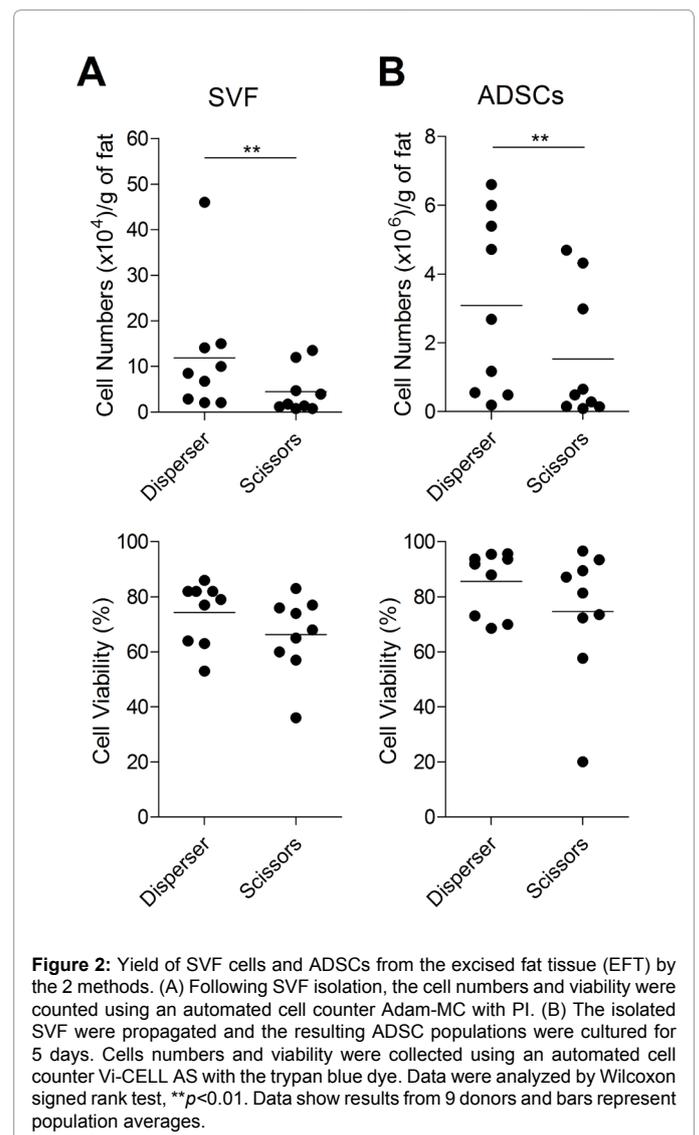

Figure 2: Yield of SVF cells and ADSCs from the excised fat tissue (EFT) by the 2 methods. (A) Following SVF isolation, the cell numbers and viability were counted using an automated cell counter Adam-MC with PI. (B) The isolated SVF were propagated and the resulting ADSC populations were cultured for 5 days. Cells numbers and viability were collected using an automated cell counter Vi-CELL AS with the trypan blue dye. Data were analyzed by Wilcoxon signed rank test, **$p<0.01$. Data show results from 9 donors and bars represent population averages.

cell viability was 0.8-4.8 folds higher (Figure 2B). Although the cell viability did not significantly differ between the two methods for both SVF and ADSCs (p=0.25, p=0.3594, respectively), the total yields were significantly higher for both SVF and ADSCs using the tube disperser method (p=0.0039, p=0.0039, respectively; Figure 2).

ADSCs expansion in different culture media

We compared the growth of ADSCs in the medium containing 10% HAS and 10% FBS, and found that the growth is slower in the 10% HAS-supplemented medium (Figure 3B). To improve the growth of ADSCs without the use of animal serum, we added two antioxidants (NAC and AsA2P) to the HAS-supplemented medium, where FGF-2, NAC and AsA2P supplements were collectively named MCA as previously described [37]. The morphology and growth of ADSCs between the following conditions were compared: 10% FBS, 10% HAS, 10% HAS+MCA and 2% HAS+MCA. ADSCs were found to maintain their spindle-shaped morphology under all conditions on day 3 (Figure 3A). ADSC expansions in the 10% HAS+MCA condition significantly increased from 155% to 324% between days 1 to 7 as compared to the 10% HAS condition (Figure 3C). However, ADSC expansions in the 2% HAS+MCA condition markedly decreased when compared to the 10% HAS+MCA condition (Figure 3D).

Karyotyping, stemness genes, stem cell-related genes, and growth factor genes of ADSCs

Karyotypes of ADSCs from 2 donors after cultured in 10%

Figure 3: The growth of ADSCs prepared by the tube dispersion method cultured with different media. (A) Direct visualization of cell culture and cell morphology on day 3. Bar=500 μm. ADSC proliferation were examined by a time course of cell densities under the 10% FBS, 10% HAS, 10% HAS+MCA and 2% HAS+MCA conditions between days 0-7. (B) 10% FBS vs. 10% HAS. (C) 10% HAS+MCA vs. 10% HAS alone. (D) 2% HAS+MCA vs. 10% HAS alone. Data are shown as mean ± SD (n=3). *p<0.05. **p<0.01. ***p<0.005.

HAS+MCA-supplemented medium for 1 month were shown in figure 4A and 4B. No abnormality was observed. The expression of 2 stemness genes (Figure 4C), 2 stem cell-related genes (Figure 4D), and 3 growth factors (Figure 4E) of ADSCs maintained in various culture supplements were compared. The expression of *Nanog*, *SOX2* (sex-determining region Y-box 2), *CXCR4* (C-X-C chemokine receptor type 4), *TERT* (telomerase reverse transcriptase), *IGF-1*, *HGF*, and *EGF* were enhanced in the 10% HAS-, 10% HAS+MCA- and 2% HAS+MCA-supplemented media as compared to those in the 10% FBS-supplemented medium (Figure 4C and 4D).

Osteogenic, chondrogenic and adipogenic differentiations

Following culturing in differentiation-inducing media, the differentiation status of ADSCs was assessed. Alkaline phosphatase staining (Figure 5A) and von Kossa staining (Figure 5B) revealed the osteogenic differentiation of ADSCs. Expression of several osteogenic genes (i.e. *cbfa 1*, *OC* and *COL IA1*) after osteogenic induction was also studied (Figure 5C). In general, the expression of these genes increased for all HAS-containing conditions as compared to those in the FBS-supplemented medium. Sulfated proteoglycan-rich matrix (alcian blue staining), indicating chondrogenic differentiation, was the highest in ADSCs cultured in the 2% HAS+MCA condition (Figure 6A). Expressions of *ACAN* and *COL IIA1* increased significantly in ADSCs cultured in the 10% HAS+MCA- and 2% HAS+MCA-supplemented media (Figure 6B). Oil Red O-positive lipid vacuoles, indicating adipogenic differentiation, were evident in ADSCs for all conditions (Figure 6C). The expression of *PPARγ* increased in ADSCs cultured in the 10% HAS+MCA- and 2% HAS+MCA-supplemented media, while the expression of *aP2* was increased only in the 10% HAS+MCA condition (Figure 6D). Thus, following the addition of differentiating media, ADSCs conditioned in both HAS and MCA further promoted all three types of differentiation when compared to ADSCs conditioned in media supplemented with FBS or HAS alone.

Surface markers of ADSCs

We have examined the phenotype of ADSCs grown in different culture conditions at passage 1 (Figure 7A) and passage 2 (Figure 7B) for the identification of MSCs. The expression levels of CD44 and CD73 were higher on ADSCs grown in the HAS-containing media (10% HAS, 10% HAS+MCA or 2% HAS+MCA) compared with those grown in the FBS-supplemented medium during passage 1, whereas the expression level of CD90 was lower on ADSCs grown in all HAS-containing media as compared with those grown in the FBS-supplemented medium. In addition, only ADSCs grown in the FBS-supplemented medium expressed CD34 at passage 1. These differences in expression levels between the HAS- and FBS-containing media were small but steady during passage 1. However, no differences in the expression of these markers were found during passage 2.

Cytokine profiles of ADSCs

Finally, we used a commercially available antibody array to compare the secretion of 41 growth factors between the supernatant of ADSCs grown in the 10% HAS- and 10% HAS+MCA-supplemented media for 4 days. FGF-2, epidermal growth factor (EGF), platelet-derived growth factor (PDGF) isoforms AA, AB and BB, and vascular endothelial growth factor (VEGF) R3 in the supernatant decreased after 4 days of culture in the 10% HAS condition. Hepatocyte growth factor (HGF), insulin-like growth factor binding proteins (IGFBP)-1, IGFBP-4, IGFBP-6, insulin-like growth factor (IGF)-1 and VEGF
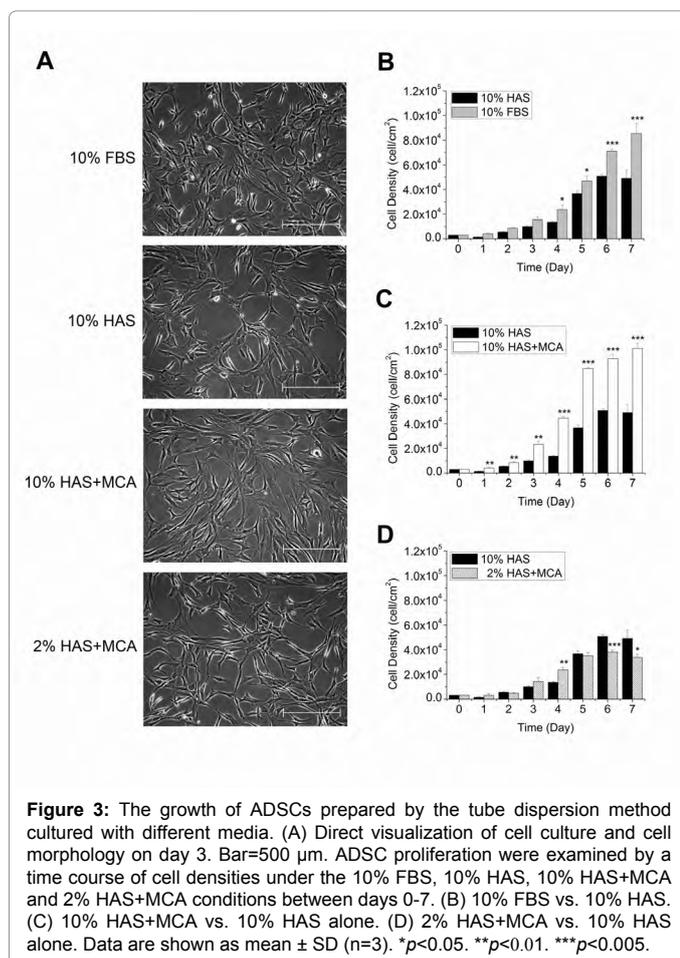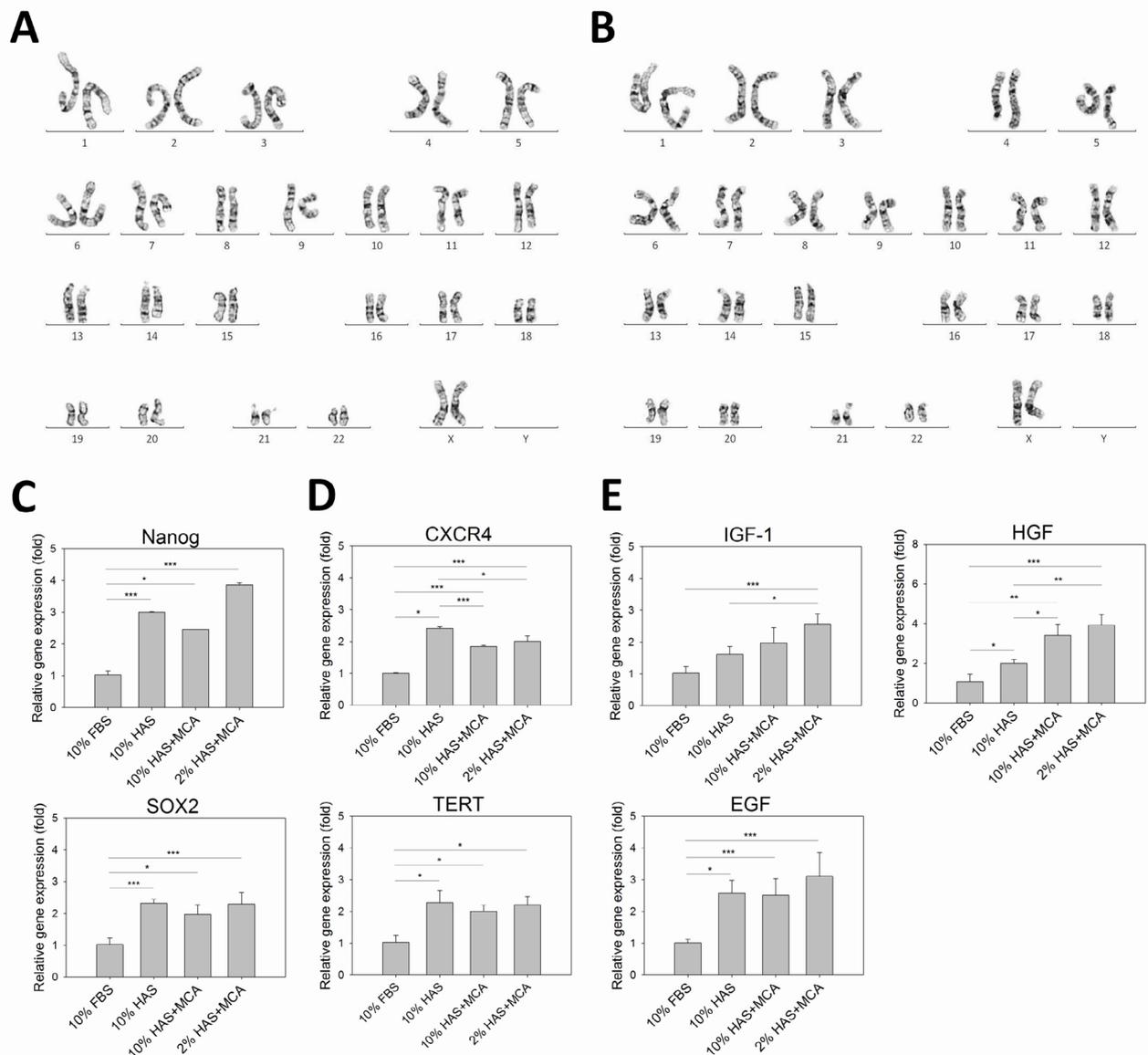
Figure 4: Karyotype and the expression of stemness genes and growth factor genes of ADSCs. (A, B) Karyotyping analyses were carried out using ADSCs from donors 3 and 8 following culturing in 10% HAS+MCA-supplemented medium for 1 month. Quantitative PCR were performed to quantify the relative gene expression of (C) the stemness genes: *Nanog*, *SOX2*, (D) the stem cell-related genes: *CXCR4*, *TERT*, and (E) the growth factor genes: *IGF-1*, *HGF* and *EGF* in the ADSCs that were cultured in the different culture media for 7 days (n=3). The gene expression was normalized to *β-actin*, serving as the internal control, and made relative to the expression levels of each gene measured for cells cultured under the 10% FBS condition. Each bar represents means ± SD. *$p<0.05$. **$p<0.01$. ***$p<0.005$.

increased after 4 days of culture in the 10% HAS condition (Figure 8A and 8B). Comparing between the 10% HAS and 10% HAS+MCA conditions, PDGF-AA, PDGF-AB and PDGF-BB content found in the supernatant decreased in the 10% HAS+MCA condition, while the VEGF R3 increased. At the same time, the secretion of HGF and VEGF increased in the 10% HAS+MCA condition and the secretion of IGF-1 and IGFBP-4 decreased (Figure 8C). These results indicate that MCA can affect the autocrine and/or paracrine activities of the ADSCs in the microenvironment.

Discussion

The development of GTP/GMP processes for the isolation of SVF

or ADSCs using small amount of EFT represents a major advance in the fields of cell therapy and tissue engineering. However, the current manual operation with surgical scissors in EFT processing has serious limitations. To minimize these limitations, we developed a semi-automated tissue processing method for the isolation of SVF (Figure 1). This semi-automated process has three important advantages. First, the new process can markedly reduce the operation time for mincing tissues from one hour down to a few minutes. Second, the yields of SVF and ADSCs can be increased by the semi-automatic protocol. Third, the use of a disposable disperser tube instead of surgical scissors (requiring repeated disinfection and aseptic validation) eliminates a potential source of contamination. To the best of our knowledge, this
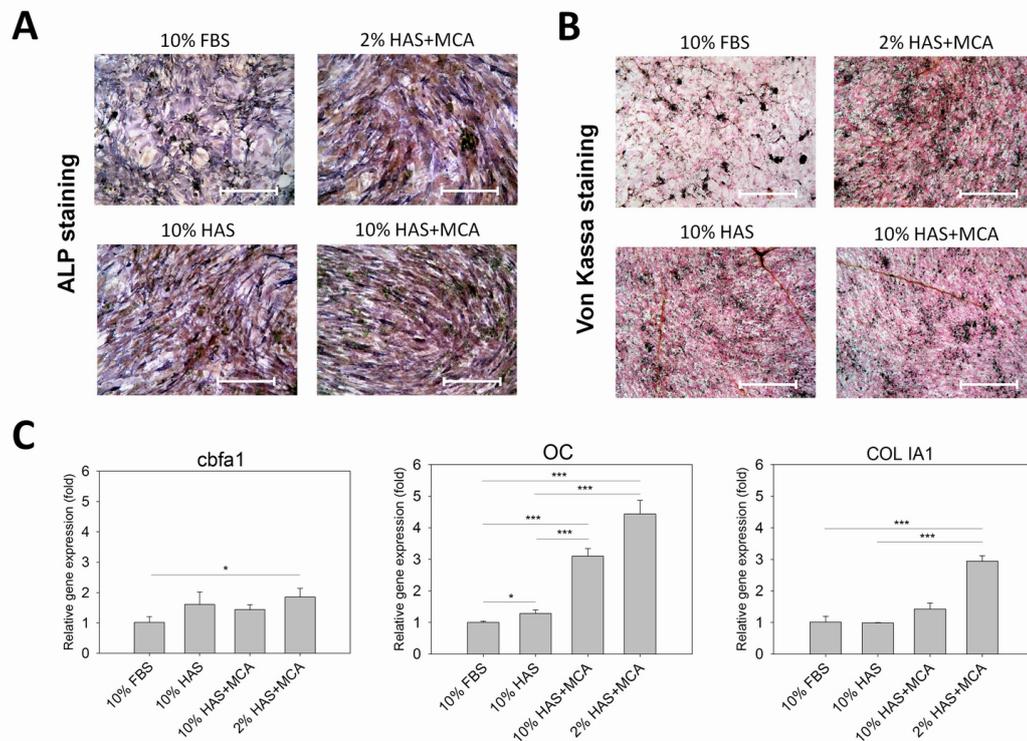
Figure 5: The osteogenic differentiation of the ADSCs. After incubation in the different culture media for 6 days (n=3), the ADSCs were then exposed to the differentiation medium for 14 days and examined for their osteogenic differentiation. Photomicrographs show alkaline phosphatase (ALP) activity (A) and von Kassa staining (B) of the ADSCs. Bar=500 μm. Both ALP and von Kassa staining demonstrate osteo-differentiation of the ADSCs. (C) The expression of osteogenesis-associated genes in the ADSCs quantified by quantitative PCR analyses, using β-actin as the internal control. Each bar represents mean cell density ± SD. *$p < 0.05$. **$p < 0.01$. ***$p < 0.005$.
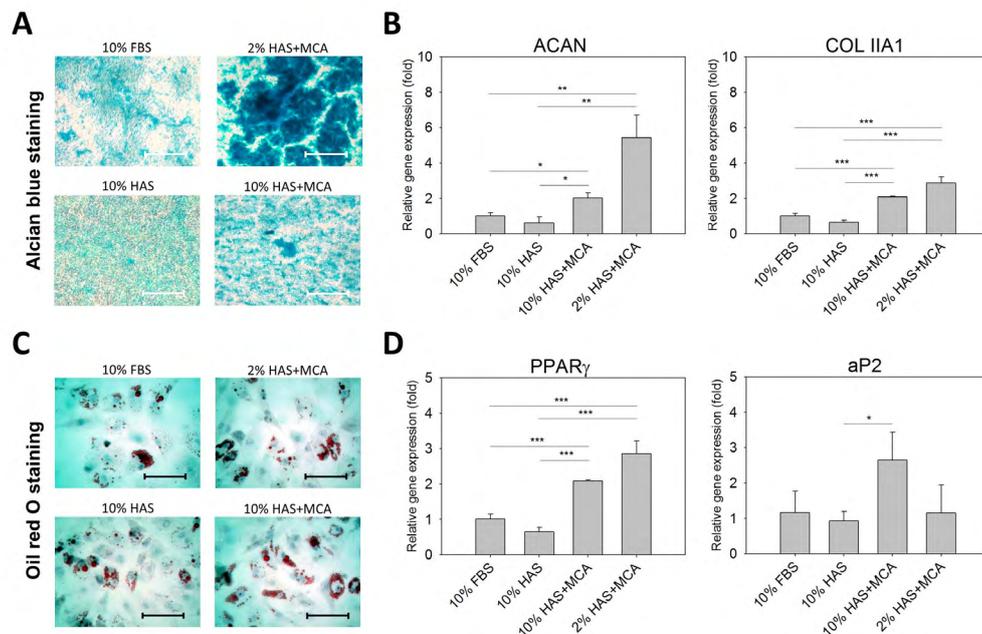

Figure 6: Chondrogenic and Adipogenic differentiation of the ADSCs. After incubation under the different culture media for 6 days (n=3), the ADSCs were then exposed to the chondrogenesis and adipogenesis differentiation medium for 14 and 7 days, respectively. (A) Photomicrographs show sulfated proteoglycan-rich matrix (blue color) indicating chondrogenic differentiation in the ADSCs at day 14. (B) The expression of chondrogenesis-associated genes (*ACAN and COL IIA1*) at day 14. (C) Photomicrographs show lipid spheres (by Oil Red O staining) indicating adipogenic differentiation in the ADSCs at day 7. (D) The expression of adipogenesis-associated genes (*PPARγ* and *aP2*) in the ADSCs at day 7. For (A/C), bar=500 μm. For (B/D), β-actin was the internal control and each bar represents mean cell density ± SD (n=3). *$p < 0.05$. **$p < 0.01$. ***$p < 0.005$.
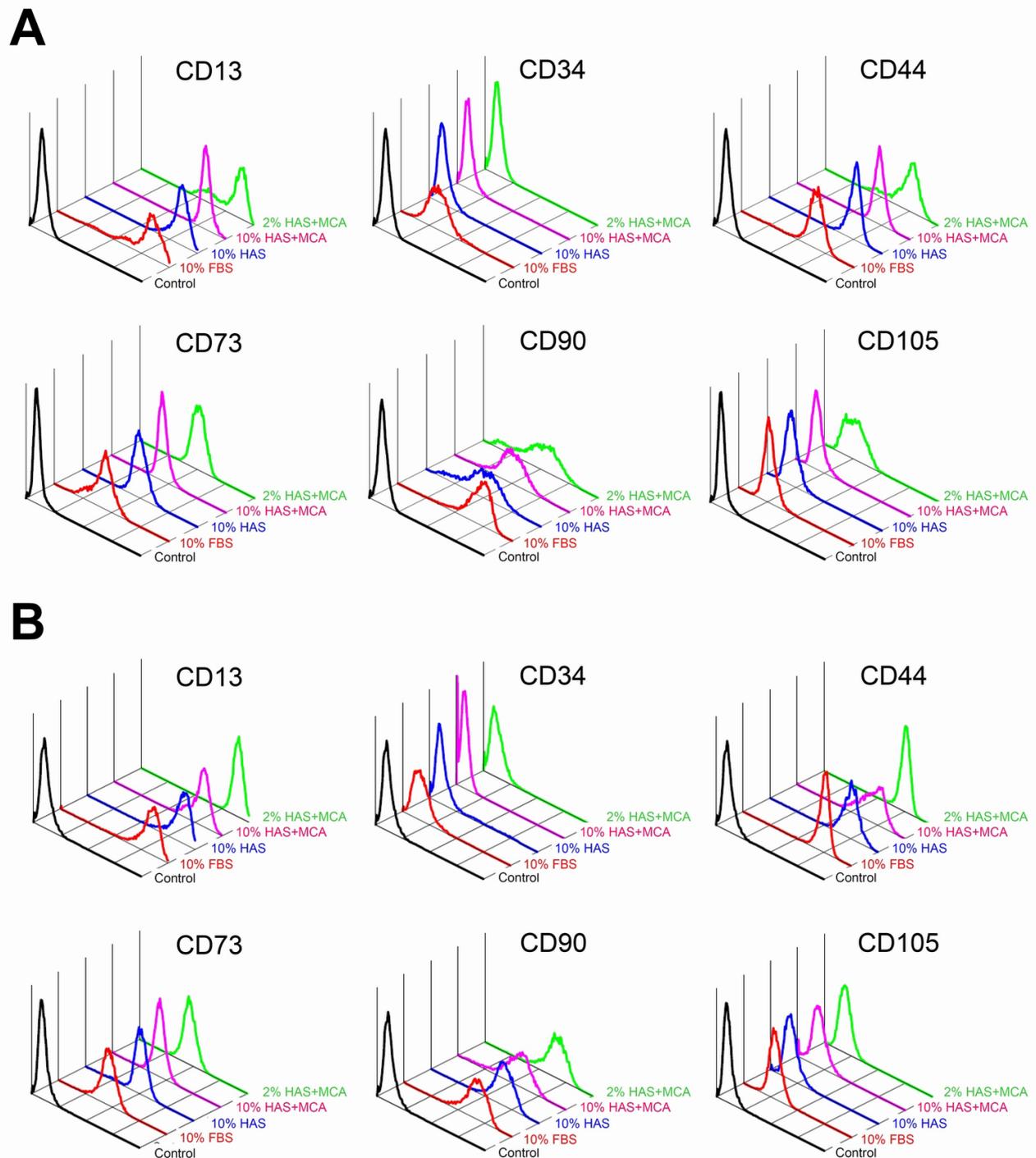
Figure 7: The phenotype of ADSCs at day 6 following culturing in respective culture media. Black lines indicate background (control) signals; colored lines represent the expression of each indicated cell surface markers during (A) passage 1, and (B) passage 2.

is the first description of a semi-automated system to manufacture ADSCs from EFTs.

Since only very small amounts (1%–5%) of SVF cells isolated from

AFT will become ADSCs, liposuction is not ideal for individuals with low BMI [7,9]. For these individuals, an enhanced SVF and ADSC extraction efficiency is required for effective cell therapy, where direct
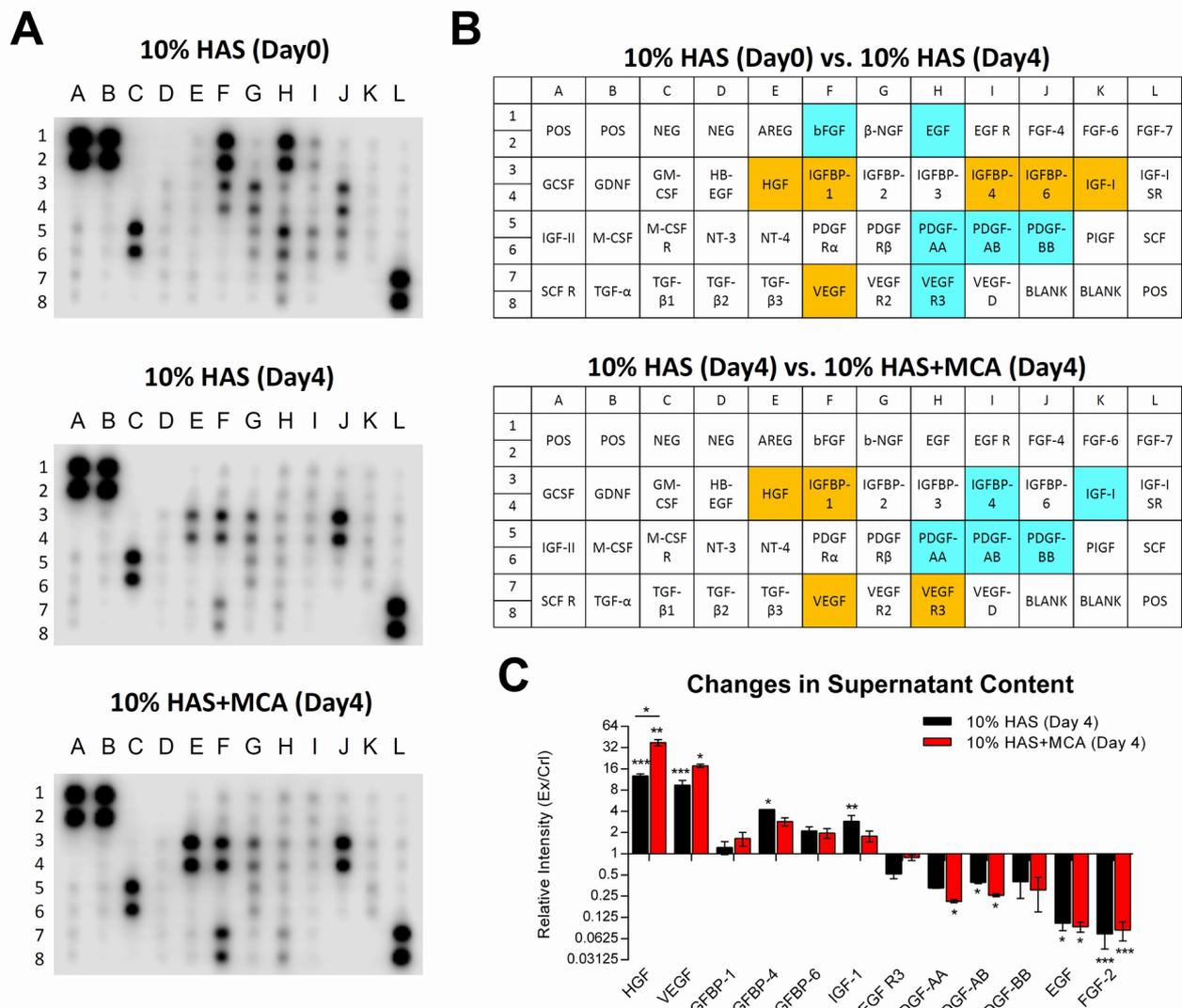
Figure 8: The cytokine profiles of the ADSCs exposed to the different culture media (n=3). Supernatants harvested from the cultured ADSCs were compared by cytokine array on day 4. (A) The cytokine arrays. (B) Map of the cytokine arrays. Blue indicates reduction in the supernatant and orange indicates cytokine secretion. (C) Relative ratio of cytokine expression levels from cytokine array membranes. Cytokine and growth factor expression from culture supernatants were normalized against an internal positive control (POS) on the same membrane and presented as the relative intensity ratio of cultured, experimental (Ex) groups at day 4 to uncultured, control (Crl) group at day 0 (Ex/Crl). Data are presented as mean ± SD (n=3). The relative ratios of less than 1 are reduction of content in the supernatant, and the relative ratios of greater than 1 are classified as secretion. *$p<0.05$. **$p<0.01$. ***$p<0.005$.

excision of fat tissues has been found previously to increase cell yield [6]. Thus, processing of EFT by our semi-automatic protocol is inevitably necessary. Our study demonstrated that the total SVF cell yield of EFT (n=9) was $2.65 \pm 2.2 \times 10^5$/ml by surgical scissors and $3.53 \pm 2.2 \times 10^5$/ml by tube dispersers. The cell viability was $66.22 \pm 14.12\%$ by surgical scissors and $74.22 \pm 11.36\%$ by tube dispersers, indicating that the semi-automatic method enhances the viable cell yields. Studies regarding the total SVF yield from both EFT and AFT are rarely reported in the literature. Although one study has reported total SVF cell yields of $1.44 \pm 1.62 \times 10^6$/ml from males (n=36) and $2.05 \pm 1.46 \times 10^6$/ml from females (n=78) [2], SVF yields obtained from AFT in our laboratory was $3.28 \pm 5.59 \times 10^5$/ml (n=11) (data not published). The differences in cell yield may be due to differential cell counting methodology, where

the large amount of red blood cells present in the SVF samples are more easily excluded using Propidium Iodide (PI) instead of the Trypan Blue dye. In comparison to the cell yields obtained from our AFT samples, cell extractions from EFT samples by our semi-automated method, on average, lead to higher cell yields. At the same time, fat tissue extraction by EFT is more suitable for individuals with low BMI.

Serum is the most variable factor in the expansion of ADSCs at clinical grade standard. The use of animal sera, such as FBS, in culture medium increases the risk of transmitting zoonotic or other infectious agents [20-22]. On the other hand, the cost of proprietary chemically-defined, xeno-free SFM presents another barrier to the production of ADSCs for wide clinical applications. In fact, the clinical effects of SFM remain unclear. Some replacements of FBS such as pooled human

serum, HAS or platelet lysate (PL) have been considered as alternatives for producing MSCs in clinical applications [24,40-42]. Although HAS exhibits batch-to-batch variability, it is still the safest option due to the elimination of the risk of contamination from xenogeneic or allogeneic products. In substitution for FBS, HAS and/or PL has been tested for ADSC cultures [24,41]. However, HAS and/or PL supplement may cause cell growth arrest and even cell death [20,41]. We had similar observations where HAS supplementation alone resulted in ADSC growth arrest on day 7 (Figure 3B), but the addition of MCA supported, and increased, the continual growth of ADSCs to the 7th day (Figure 3C).

Cells must be retrieved in sufficient numbers for therapeutic efficacy [2,43,44]. In our previous study, we demonstrated that MCA promoted ADSCs to the S phase in the cell cycle by suppressing CDK inhibitors, p21 and p27, and resulted in rapid cell proliferation similar to that observed under hypoxic conditions [35]. In the present study, we found that the 10% HAS+MCA-supplemented medium enhanced ADSCs proliferation *in vitro* as compared to the 10% HAS condition (Figure 3C). The addition of MCA also reduced the amount of HAS necessary for ADSC expansion (Figure 3D). Thus, our formulation facilitates rapid ADSC expansion for individuals who require urgent autologous cell transplantation.

In our previous study, medium containing FBS and MCA caused significant reduction in the expression of CD29, CD90 and CD105 in ADSCs, while at the same time enhanced their osteogenic and adipogenic differentiating potentials [35]. In the present study, we did not find significant differences in the expression level of surface markers on ADSCs grown in media supplemented with either HAS alone or HAS+MCA at passage 1 or 2. However, the expression levels of CD34, CD44 and CD90 on ADSCs grown in the HAS-supplemented medium were different to those cultured in the FBS-supplemented medium at passage 1. The expression levels of surface markers on ADSCs have been demonstrated to be associated with the differentiation potential of ADSCs [45,46]. The higher expression of CD44 (TGF-β receptor) on ADSCs grown in the HAS-supplemented medium may explain the enhanced osteogenic (Figure 5) and chondrogenic potentials (Figure 6) [47].

ADSCs and their secretory factors such as VEGF, HGF and FGF-2 are known to be beneficial in skin repair and regeneration [48]. While hypoxia has been found to regulate the production of a variety of growth factors and enhance the paracrine effect of ADSCs to promote hair growth [49], we have found that MCA may also affect the paracrine and/or autocrine activities of ADSCs via changes of cytokine and growth factor content in the microenvironment (Figure 8). Our results showed that the 10% HAS+MCA supplement induced the reduction of PDGF-AA, PDGF-AB and PDGF-BB found in the supernatant during ADSCs proliferation that may be due to an enhanced uptake, binding to cell surface receptors, or degradation of cytokines, whereas the secretion of HGF and VEGF were enhanced (Figure 8C). To the best of our knowledge, this is the first report demonstrating that MCA can regulate the autocrine and/or paracrine activities in ADSCs.

We have developed a semi-automated process that is particularly suitable for isolating human ADSCs from small amounts of EFT and formulated a xeno-free medium using HAS (2 or 10%) and MCA for their efficient expansion. The use of HAS and chemically-defined MCA comply with the GTP and GMP requirements for human tissues and cell products, respectively. These rapidly proliferating ADSCs have normal karyotypes and typical surface marker profiles, while maintaining stemness gene expression and differentiation potential. In summary, the combination of this novel semi-automated system with the HAS+MCA-supplemented medium may provide a better solution for the clinical applications of small amounts of EFT for regenerative medicine and tissue engineering.

Competing Interests

The authors declare that they have no competing interests.

Conflict of Interest

The authors report no conflicts of interest and are responsible for the content and writing of the paper.

Funding

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