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Human Bone Marrow Stromal Cell Confluence: Effects on Cell Characteristics and Methods of Assessment

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Abstract

Background Aims—Ex vivo expansion and serial passage of human Bone Marrow Stromal Cells (BMSCs, also known as bone marrow-derived mesenchymal stem cells) is required to obtain sufficient quantities for clinical therapy. The BMSC confluence criteria used to determine passage and harvest timing vary widely and the impact of confluence on BMSC properties remains controversial. The effects of confluence on BMSC properties were studied and confluence-associated markers were identified.

Methods—BMSC characteristics were analyzed as they grew from 50% to 100% confluence including viability, population doubling time (PDT), apoptosis, colony formation, immunosuppression, surface marker expression, global gene expression and microRNA expression. In addition, culture supernatant protein, glucose, lactate and pH levels were analyzed

Results—Confluence-dependent changes were detected in the expression of several cell surface markers, 39 culture supernatant proteins, 26 microRNAs and 2078 genes. Many of these surface markers, proteins, microRNAs and genes have been reported to be important in BMSC function.

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Disclose of Interests

The authors have no commercial, proprietary or financial interest in the products or companies described in this article.

Supplementary Material

S1. List of BMSCs used for each experiment

S2: Representative figures of 50%, 80% and 100% confluence.

S3: Table showing concentrations of proteins in BMSC supernatant.

The PEDF/VEGF ratio increased with confluence, but 80% and 100% confluent BMSCs demonstrated a similar level of immunosuppression of mixed lymphocyte reactions. In addition, changes in lactate and glucose levels correlated with BMSC density.

Discussion—BMSC characteristics change as confluence increases. 100% confluent BMSCs may have compromised pro-angiogenesis properties, but may retain their immunomodulatory properties. Supernatant lactate and glucose levels can be used to estimate confluence and ensure consistency in passage and harvest timing. Flow cytometry or microRNA expression can be used to confirm that the BMSCs have been harvested at the appropriate confluence.

Keywords

Bone marrow stromal cells; BMSCs; cellular therapy; gene expression profiling; confluence; microRNA

Introduction

Bone marrow stromal cells (BMSCs), which are also known as mesenchymal stem cells (MSCs), were first isolated from the bone marrow as the progeny of colony-forming-unit fibroblasts (CFU-Fs)[1, 2]. Since BMSCs include skeletal stem cells, they have been used in clinical trials to treat osteonecrosis of the femoral head[3], osteoarthritis/cartilage repair[4, 5] and degenerative disc disease[6]. BMSCs also have anti-inflammatory and immunosuppressive properties *in vitro* and may hold promise in treating immune diseases, acute Graft-versus-Host-Disease (aGvHD)[7], Crohn's disease[8] and in preventing the rejection of transplanted solid organs[9] . BMSCs therapy has proven to be safe and capable of improving the clinical performance and the quality of life of patients with ischemic cardiomyopathy (ICM)[10]. The mechanisms underlying the ability of BMSC to improve heart function could be that MSCs differentiate into cardiomyocytes, vascular smooth muscle and endothelial cells[11] ; secrete cell survival factors including VEGF, bFGF, IGF and SDF[12] or stimulate the proliferation of endogenous cardiac stem cells (CSCs)[13] .

For clinical purposes, BMSCs are isolated from bone marrow aspirates or bone biopsy specimens and are expanded extensively ex vivo to obtain adequate numbers of cells. Most expansion protocols involve adherent culture on plastic surfaces and serial passage. Many variables must be considered when optimizing BMSC expansion, among which cell confluence is a critical factor since the degree of confluence may affect the biological properties of BMSCs. Generally, BMSCs are sub-cultured or harvested when they reach a specified degree of confluence, but there is no universal standard concerning optimal confluence. For example, investigators have used different criteria to describe the conditions when the cells need to be sub-cultured, such as 50–60% confluence[14, 15], 70–90% confluence[16], sub-confluent (70–80%)[17], 80% confluence[18–21], 80–90% confluence[22], 90% confluence[23, 24], near confluence[25], approaching confluence[26] or confluent[27–30]. More importantly, the outcomes of BMSC clinical trials have been variable. Since BMSCs used to treat GVHD and other conditions are cultured to variable levels confluence it is important to better understand how confluence at the time of harvest effects the properties of BMSCs [31].

As a Good Manufacturing Practice (GMP)-compliant laboratory, we aim to achieve optimal expansion of BMSCs by establishing the best seeding density and timing of passage and harvest in order to maintain consistent BMSC quality. When sub-culturing or passaging BMSCs in small flasks, the cells are easily observed and confluence assessed under a microscope, but when BMSCs are cultured under large scale GMP conditions they are most often cultured in multi-layer flasks or cell factories and cannot be assessed under microscope. For cell expansion under these circumstances, developing a measure to determine BMSC confluence is in urgent need.

In this study, we grew the BMSCs to 50%, 80% and 100% confluence, which were determined by the proportion of culture area that was covered by BMSC, then investigated the impact of confluences on BMSC phenotype including cellular proliferation, apoptosis, surface marker expression, growth factor and cytokine production, immunosuppressive activity, microRNA expression profile, and gene expression profile. We found multiple changes in BMSCs as their confluence increased from 50% to 100%.

Materials and Methods

BMSC Culture

Bone marrow collection and BMSC culture were performed according to Standard Operating Procedures (SOP) established in our lab[32]. These studies were approved by the NHLBI Institutional Review Board (IRB) under clinical protocol 10-CC-0053, which was also registered on clinicaltrial.gov under NCT01071577.

For the primary culture, 5 to 10 mL of bone marrow was collected from the posterior iliac crest of healthy donors after obtaining informed consent. The marrow was collected in Bone Marrow Prep Syringes (Pharmacy Department, NIH, Bethesda, MD) and then washed with 2.5x volume of HBSS (Lonza, Walkersville, MD). The cells were then re-suspended with BMSC culture media (BMSC CM) [alpha MEM with 2 mM glutamine (Lonza), supplemented with 20% lot-selected FBS (Hyclone, Thermo Fischer Scientific, Waltham, MA) and 10 μg/ml Gentamicin] and plated at a density of 2 x $10^5/cm^2$ in T-75 flasks (Corning Life Sciences, Corning, NY). The cells were incubated at 37°C in 5% CO_2 and non-adherent cells were removed after 24 hours. The media was changed every three days thereafter until the colonies reach 80% confluence. The primary BMSCs were washed with 10mL HBSS twice, digested with 5mL TrypLE Express (Invitrogen, Life Technologies, Grand Island, NY) and centrifuged at 406 x g for 10 minutes. The cell number was then counted and viability was assessed using the Trypan Blue exclusion method. The cells harvested at this stage were designated as Passage 1 and serial passage numbers were designated thereafter.

Passage 2 BMSCs from 7 donors were thawed from a working cell bank and plated at a density of 3 x $10^3/cm^2$ in T-75 flasks, the donors of these cells that were used for each experiment in this study were listed in supplemental file 1. The BMSCs were cultured to 80% confluence and harvested; the cells at this stage were called passage 3 and were plated to T75 flask for further culture. When passage 3 BMSCs reached 50%, 80% and 100% confluence as determined by microscope observation by 3 trained investigators (the

representative figures of 50%, 80% and 100% confluence were shown in supplemental file 2), they were harvested as passage 4 cells. The cell densities were $1.34 \times 10^4 \pm 5.23 \times 10^3$, $3.28 \times 10^4 \pm 8.81 \times 10^3$ and $4.58 \times 10^4 \pm 2.40 \times 10^4$ cells per cm^2 under 50%, 80% and 100% confluence respectively (Figure 1A). The BMSCs were then subjected to assays including surface marker expression, apoptosis, cell proliferation, and suppression on the proliferation of mixed lymphocytes. The culture supernatant of BMSCs at different confluences were also collected and used to measure the concentrations of cytokines and growth factors. Total RNA was extracted from an aliquot of the cells for global microRNA and gene expression profiling.

Cell Proliferation, Viability and Colony Formation Efficiency (CFE) Assays

When passage 3 BMSCs reached 50%, 80% and 100% confluence, they were harvested (passage 4), the cell number was counted and viability was measured by the Trypan Blue exclusion method. For proliferation analyses, passage 4 BMSCs from the 3 confluences were further plated in T75 flasks at a density of $3000/cm^2$ and cultured for 5 days and harvested. The proliferation of BMSC was determined by cell counting, Population doubling (PD) and population doubling time (PDT) was calculated by the method described previously[33]. For CFE assays, passage 4 BMSCs from 50%, 80% and 100% confluences were plated at $1.67/cm^2$ in T25 flasks in duplicate and cultured for 13 days without changing culture medium. The colonies were then fixed with methanol for 30 minutes and stained with saturated methyl-violet water solution for 20 minutes. Colonies (>50 cells) were then counted under low magnitude light microscope field (25 X).

Apoptosis Analysis by Flow Cytometry

The rate of apoptosis of 50%, 80% and 100% confluent BMSCs was measured with Annexin V-Apoptosis kit (BD Bioscience, San Jose, CA). Briefly, cells were washed twice with cold PBS, re-suspended in binding buffer and then incubated with FITC-labeled Annexin V for 15 minutes at room temperature in the dark. Afterwards, the cells were washed and stained with Propidium Iodide (PI); the cell counts were acquired on an Accuri C6 flow cytometer (Accuri, Ann Arbor, MI) and data analyzed by CFlow software (Accuri), Annexin V-positive and PI-negative cells were enumerated as apoptotic cells.

Flow Cytometry Analysis of Surface Marker Expression

Cell surface markers were measured on 50%, 80% and 100% confluent BMSCs by Flow Cytometry assay; the markers were selected according to either global gene expression profiling results or from the literature[34–36]. The following antibodies were used, PODXL-FITC (MBL International Inc, Nagoya, Japan), CD49f-PE, CD54-APC, TLR1-PE, CD106-PE, LAMP1 (CD107a)-APC, CD146-PE, CCR7-PE, CD55-FITC, TLR4-PE (BD Biosciences), CD200-APC, CD10-APC (Biolegend, San Diego, CA, USA), and FZD4-APC (R&D, Minneapolis, MN, USA), isotype controls were IgG1-FITC, IgG1-PE, IgG2a-FITC (BD Bioscience), IgG1-APC (eBioscience, San Diego, CA, USA). In brief, BMSCs were incubated with antibody cocktail for 30 minutes in the dark at room temperature and washed with PBS containing 1% BSA; the cells were then suspended in 0.3 mL PBS with 1% BSA, counterstained with 7-AAD; twenty-thousand events were acquired on an Accuri C6 flow cytometer. The data were analyzed with FlowJo software (TreeStar, Ashland, OR). Positive

cells were identified as those whose intensities were greater than 99 percentile of the isotype control.

Measurement of Glucose and Lactate, Growth Factors and Cytokines in Culture Supernatants

The concentrations of glucose and lactate in the culture supernatant of BMSC were measured daily by using an i-STAT Analyzer (Abbott, Princeton, NJ). The raw data were imported into Excel spreadsheets; glucose consumption rate (GCR) and lactate generation rate (LGR) were calculated by dividing the time when the consecutive measurements were completed. The concentrations of cytokine and growth factors in BMSC supernatants were measured with SearchLight Protein Array (Aushon Biosystems, Billerica, MA). Briefly, supernatants were collected daily and centrifuged for 10 min at 1400 rpm, aliquoted and stored at −80 °C . The 64 proteins that were measured included cytokines, growth factors, chemokines, and enzymes; the list is shown in Supplemental Table 1.

Suppression of Mixed Lymphocyte Reaction (MLR)

The immunosuppressive properties of 80% and 100% confluent BMSCs were compared using MLR assay (SAIC-Frederic, Frederic, MD). Briefly, Ficoll-separated peripheral blood mononuclear cells (PBMC) were plated in 96-well plates at $1x10^5$ responders per well. Responders were co-cultured with stimulator PBMCs at the same concentration. BMSCs grown to 80% and 100% confluence were added at the following concentrations, 10^4 and 10^5 cells /well. After 6 days of culture in a humidified 5% CO_2 incubator at 37°C, 0.5 μCi of ^{3}H-thymidine (^{3}H-TdR) was added to each well for 4 hours, and the lymphocyte proliferation was measured using a liquid scintillation counter. The effect of BMSCs on MLR was calculated as the percentage of the suppression compared with the proliferative response of the positive control without BMSC, where the positive control was set to 0% suppression. The experiments were performed in triplicate.

Gene and microRNA Expression Profiling

Total RNA was extracted using miRNeasy Mini Kit (Qiagen, Hilden, Germany) and assessed using the Nano Drop 2000 (Thermo Scientific, Wilmington, DE). Total RNA (0.5 μg) was labeled with Cy^5-CTP using Quick Amp Labeling kit (Agilent, Santa Clara, CA). An equal amount of human reference RNA (Stratagen, La Jolla, CA) was labeled with Cy^3-CTP. 825 ng of labeled cRNA was pooled, fragmented and then hybridized on 4 x 44K microarrays (Agilent) for 17 hours at 65°C. The microarrays were then washed and scanned using an Agilent Scanner and data were acquired using Agilent Feature Extraction Software. The data were submitted to Gene Expression Omnibus (GEO) under accession number GSE60525.

MicroRNA profiling was performed by a protocol established in our lab[37]. Briefly, 4 μg total RNA was labeled with miRCURY™ LNA Array Power Labeling Kit (Exiqon, Woburn, MA). The total RNA from Epstein-Barr virus (EBV)-transformed lymphoblastoid cell line was used as the reference. The test sample was labeled with Hy^5 and the reference with Hy^3. After labeling, the test sample and the reference were co-hybridized to the custom-made microRNA array at room temperature overnight in the present of blocking

reagents; the slides were then washed and scanned by GenePix scanner Pro 4.0 (Axon, Sunnyvale, CA, USA). The data were submitted to Gene Expression Omnibus (GEO) under accession number GSE60449.

Data Processing and Statistical Analyses

Gene and microRNA expression analysis was performed by procedures as reported previously[37, 38]. Raw data were uploaded into mAdb database (http://madb.nci.nih.gov/) and then analyzed by BRB-ArrayTools[39] (http://linus.nci.nih.gov/BRB-ArrayTools.html) and Partek Genomics Suite (Partek Inc, Saint Louis, MO). Tests for differences between different degrees of confluence were conducted for individual genes and microRNAs using two-way ANOVA, considering P values of <0.001 as significant for genes, and <0.05 as significant for microRNAs. Clusters and Heatmaps were generated by MultiExperiment Viewer (MeV4.6.2, http://www.tm4.org/mev/)[40]. We also did functional annotation on the differentially expressed genes, aiming to identify the gene sets underlying the different degree of confluence. In this way, the genes were first organized into higher-level sets, which can be downloaded from Genomica Repository (http://genomica.weizmann.ac.il/). The confluences in which different sets are “induced” or “repressed” were identified following methods described previously[41]. The significance of the gene sets and confluences were calculated by using Genomica[41].

Results

Effect of Confluence on BMSC Proliferation, Viability, Apoptosis, CFE and Immunosuppression

All the BMSCs used in this study were derived from 7 healthy donors who were less than 44 years old and all the cells were from early passages (passage 4) in order to reduce the influence of passage as we have described previously[42]. The viability was >95% for all 3 confluences and there was no significant difference in viability among the three confluences (Figure 1B).

To address the influence of contact inhibition on cell growth, the BMSCs were harvested after becoming 50%, 80% and 100% confluent and re-seeded into T75 flasks. After 5 days of culture, the cell number was counted and the population doubling time (PDT) was calculated. For all 3 donors tested, the PDT of 80% confluent BMSCs (34.2 hrs) was slightly shorter than 50% confluent cells (39.3 hrs, p=0.22), but much shorter than 100% confluent cells (46.8 hrs, p=0.02) (Figure 1C).

We also measured apoptosis using an Annexin V Flow Cytometry assay, in which the apoptotic cells are positive for Annexin-V but negative for propidium iodide (PI). All the BMSC samples displayed low apoptosis rate (<3%), and there was no difference among the 3 degrees of confluence (Figure 1D).

Colony Formation Efficiency (CFE), is a good measure of BMSC quality, since only a proportion of the BMSCs can re-adhere to plastic surfaces and proliferate in a density independent fashion. To determine the influence of 100% confluence, we performed CFE assays and documented the quantities of the two types of colonies that grew (Figure 1E); one

type was the rapidly growing (RG)colonies, which were composed of fibroblastic cells with extended cell processes that formed dense colonies, the other was the slowly growing (SR) colonies, which were composed of larger cells and formed sparse colonies[43]. Our analysis revealed similar quantities of total CFE from 80% and 50% confluent BMSCs, but less from 100% confluent BMSCs. When we enumerate the two types of colonies, 50% confluent BMSCs produced the highest percentage of RS colonies, while 80% and 100% confluent cells yielded similar percentages of RS colonies, but the difference was not statistically significant among the three degree of confluence (Figure 1F).

To assess the immunomodulatory effects of the BMSCs their ability to inhibit mixed lymphocyte reactions was compared among 80% and 100% confluent cells from 3 donors. Two doses of BMSCs, 10,000 and 100,000 cells per well, were tested. At the lower dose of 10,000 cells per well (BMSC: PBMC ratio=1:10), the immunosuppression was very similar between 80% and 100% confluent BMSCs. At the higher dose of 100,000 cells per well (BMSC: PBMC ratio=1:1), 80% confluent BMSCs were slightly more suppressive than 100% confluent BMSCs (Figure 1G), but the difference was not statistically significant ($p=0.11$).

Proteins in BMSC Culture Supernatants

A total of 64 proteins, including cytokines, growth factors, chemokines and enzymes, were measured in the supernatant of 50%, 80% and 100% confluent BMSCs from 4 donors. Among these 64 proteins, 10 were undetectable, 15 were below the limit of detection, and 39 proteins were detected during the culture period (Supplemental File 3). The baseline levels of these 39 proteins in the culture medium (without contact with cells) were undetectable or very low except for TGFB1, PEDF and TSP1. At the initiation of cell culture (Day 0), the concentrations of most proteins were low, except for PEDF, TGFB1, TGFB2, TIMP1 and FN1. Afterwards, the protein concentrations increased daily during the culture (Figure 2A and Supplemental Table 1). To decide whether these increases were caused only by the increasing number of cells or by the increasing number of cells plus increasing protein production per cell, we normalized the factor concentrations by cell number. We found that protein concentrations were not linearly correlated with the cell number, thus suggesting cell-cell contact caused by confluence changed the quantities of proteins secreted. The cell number-normalized protein concentrations displayed distinct patterns; some were either steadily increasing or steadily decreasing, while some were bi-phasic (Figure 2B). For example, PEDF, IGFBP2, IGFBP3 and MMP2 displayed a steadily increasing product pattern (a representative plot of PEDF is shown in Figure 2C). In contrast ANG1, ANG2, TGFB2, GDNF, BDNF, BNGF, OPG, ICAM1 and PLGF showed a steadily decreasing pattern (i.e., ANG1 in Figure 2C). Finally, Endoglin (ENG), IL6, IL8, OPN, FN1 and TSP1 showed a bi-phasic pattern (i.e., Endoglin in Figure 2C)

Effects of Confluence on BMSC Surface Marker Expression

The expression of BMSC surface markers were also affected by confluence. Among these markers, CD10, CD54, CD106, CD200 and TLR4 were up-regulated in 100% confluent BMSCs compared with 50% and 80% confluent cells (Figure 3), while CCR7 and FZD4 were not detected (positive percentage <1%). This suggests that CD10, CD54, CD200 and

TLR4 may serve as biomarkers of BMSC confluence. In particular, CD10, which is expressed by BMSCs as well as by mesenchymal cells from other tissues[44–46], was barely detectable at 50% and 80% confluence, but a sharp increase in its expression was observed when BMSCs reached 100% confluence (Figure 3). Of note, the expression of CD49f was down-regulated with confluence in both donors; PODXL was slightly down-regulated at 100% confluence in one donor and down-regulated at 80% and 100% confluence in another. These data were in agreement with a previous report[34]; the only difference being that a comparison was made only between low density cells and those making initial contact cells in the published paper and it is unclear what would occur when the BMSCs reached 100% confluence.

Glucose and Lactate Levels in BMSC Supernatants

One goal of this study is to discover biomarkers that can be used for monitoring BMSC confluence during the manufacturing process. To identify surrogate markers of BMSC confluence, we measured glucose and lactate levels and pH values of the BMSC culture supernatants. The pH values fluctuated daily, ranging from 7.27 to 7.81 and there was no relationship between pH and BMSC confluence (data not shown). The glucose levels decreased daily despite small fluctuations after medium change on day 3, 5 and day 7 and they were inversely correlated with cell number with correlation coefficients ranging from 0.89 to 0.96, which were significant for BMSCs for all 4 donors ($p<0.01$) (Table 1 and Figure 4A). In contrast, the lactate values increased daily and also correlated with cell number with correlation coefficients ranging from 0.88 to 0.96 ($p<0.01$, Figure 4A). We also calculated the rate of the change in glucose and lactate values noticing that both the glucose consumption rate (GCR) and lactate generation rate (LGR) were significantly correlated with cell number (Table 1 and Figure 4B). Based on these data, we concluded that the values of glucose and GCR, or lactate and LGR are good indicators of cell number and can be used to determine the timing of BMSC passage or harvesting.

MicroRNA expression profiling

The microRNA profiling of 50%, 80% and 100% confluent BMSCs was conducted on custom-made microarrays with 1920 probes. After filtering out the array spots which did not meet the criteria of size (between 20 and 300nm), presence (greater than 50%) and intensity (greater than 20), and species (mouse, rat, etc), a total of 166 human microRNAs were considered evaluable. Unsupervised hierarchical clustering using these 166 microRNAs separated the BMSC samples into distinct clusters based on their degrees of confluence: 50% confluent BMSCs were located primarily within one cluster, 100% confluent BMSCs were located within another, while 80% confluent BMSCs were segmented within either 50% confluence (2 of 5 samples) or 100% confluence (3 of 5) (Figure 4C). For further analysis, 26 microRNAs that were differentially expressed among the 3 degrees of confluence were identified by using 2-way ANOVA, considering $P<0.05$ as significant. Hierarchical clustering analysis using these 26 microRNAs separated the 15 BMSC samples into 2 major clusters; one with all five 50% confluent samples and three 80% confluent samples; another one with all five 100% confluent samples and two samples of 80% confluence. Within one cluster, the 50% confluent BMSC samples formed a tight sub-cluster when compared with the 80% confluent BMSCs, as did the 100% confluent samples (Figure

4D). Among the differentially expressed microRNAs miR-17, miR-106b, miR-138, miR-26b, miR-130b, miR-494, miR-663 and miR-638 have been previously reported to be expressed in BMSCs and may be involved with osteogeneic or chondrogenic differentiation[47–49], and miR-17, miR-29c and miR-486 may be associated with aging or senescence of BMSC[50, 51] (Table 2).

Gene Expression Profiling of BMSCs

Gene expression profiling was also performed on 50%, 80% and 100% confluent BMSCs. Among all the genes, 11,795 passed the variance criteria. Principle Component Analysis (PCA) using these genes separated the samples into 2 groups, one included 50% confluent BMSCs, while the other included 80% and 100% confluent BMSC (Figure 5A). These results were consistent with published data that BMSCs of high confluence have different gene expression profiles compared to those of low confluence[52]. We then identified 2708 genes that were differentially expressed among the 3 confluences by using 2-way ANOVA (p-value <0.001). Hierarchical clustering analysis using these genes revealed that 50% confluent BMSCs formed one cluster, while 80% and 100% confluent BMSCs formed another (Figure 5B). Among these differentially expressed genes, most were differentially expressed between 50% confluent and 80% confluent BMSCs ($p<0.001$, $FC\geq1.5$, $n=1092$), or between 50% confluent and 100% confluent BMSCs ($p<0.001$, $FC\geq1.5$, $n=1252$), while only 226 genes were differentially expressed between 80% and 100% confluent BMSCs (Figure 5C).

To obtain an overview of the functions of these differentially expressed genes, we conducted a gene set enrichment analysis. A heat-map was generated based on the significance that was defined by the percentage of hit genes with each gene set using Genomica software with default settings[41]. The significantly changed ($p<0.05$, $FDR<0.1$) gene sets, either “induced” or “repressed”, as defined by Segal[41] were grouped into 5 clusters (Figure 5D).

Most of the gene sets in cluster A were related to the metabolic process; at the individual sample level, these gene sets were repressed in 5/5 80% confluent samples, in 5 of 5 100% confluent samples and in 2/5 50% confluent samples. However, as we grouped the BMSCs samples according to their degrees of confluence, these gene sets did not differ significantly among the 50%, 80% and 100% confluent groups (Figure 5D).

The gene sets in cluster B which were repressed by 80% and 100% confluent BMSC samples included Regulation of Mitosis, Cell Cycle Checkpoint and Regulation of Cyclin-Dependent Protein Kinase Activity. These results were in accordance with published data[52].

The majority of gene sets in cluster C were induced in all the individual samples but did not differ significantly at the group of confluence level (Figure 5D, right panel). The exception was Growth Factor Activity, which was particularly induced by 100% confluence and the genes contributing to this set were IGF1, GRN, CSF1, KITLG, FGF5, VEGFC, S100A6, CLCF1 and DKK1.

The gene sets in Cluster D were either induced or repressed by 50% confluence but not by 80% or 100% confluence, interestingly, two gene sets associated with microRNAs, hsa-miR-184 and hsa-miR-205 were identified in this cluster.

Gene sets in cluster E were mainly induced by 80% and 100% confluence but not by 50% confluence. These genes included Extracellular Region, Immune Response, Cell Adhesion, Cell Migration and Inflammatory Response.

The Extracellular Region set contained a large number of genes that are functionally important in both stromal and stem cell functions. Among the genes in the Extracellular Region set that are likely involved with stromal cell functions were those encoding extracellular matrix proteins: FBN1, FBLN1, FBLN5, ECM2, LUM, DCN, COLQ, COL16A1 and COL10A1; enzymes and inhibitors: PLAU, SERPINE2, PAPPA, TIMP3, TIMP1, CTSD, ADAMTS10 and ADAMTS1; growth factors: SERPINF1 (PEGF), CSF1, KITLG, TNFSF15, FGF5, CLCF1, ANGPTL2 and IGF1 and chemokines: CCL13, CCL7 and CCL2. The Extracellular Region included stem cell genes that included Wnt signaling transducers: WNT7B, DKK1, DKK2, DKK3 and WISP1; TGF-beta signaling pathway transducers: DCN, LTBP3, BGN and HTRA1; associated with osteoblastic differentiation: TNFRSF11B, SPOCK1, BGN; and associated with chondrogeneic differentiation VCAN, COMP and COL10A1. Evaluation of the expression of the individual Wnt signaling pathway inhibitor genes DKK1, DKK2 and DKK3; the angiogenesis inhibitors SERPINF1, TNFSF15 and ADAMTS1 revealed that they were up-regulated by 100% confluent BMSCs, suggesting that 100% confluence may comprise some of the beneficial properties of BMSCs including their stem cells qualities and support of angiogenesis.

The genes contributing to Immune Response were FTH1, GPX1, CCL2, ITGB5, TNFAIP6, VCAM1, GBP2, DES, MYLK, FAP, CDH13, TIMP3, TIMP1 and CTSB. Among these genes TNFAIP6 is an important regulator of immunosuppression and anti-inflammation response[53, 54]. Moreover, genes involved in Inflammatory Response were particularly up-regulated by 100% confluence and these genes were PLA2G4C, SERPING1, ANXA1, CCL13, CCL7, CCL2, TNFAIP6, SAA2, CFHR1, CFH, IL1RAP, C1S, C1R and PRKCA.

Discussion

In the field of cellular therapy, the degree of confluence at which to passage or harvest BMSCs remains an important unanswered question. Using multiple different measures, we found that the characteristics of BMSCs change as they grow from 50% to 80% and to 100% confluence, particularly when they reach 100% confluence. Our results suggest that harvesting cells at 80% confluence is optimal since BMSC proliferation slows when they reach 100% confluence and changes in expression of biomarkers in 100% confluent cells suggests that their pro-angiogenesis activities may be compromised. We found that culture supernatant levels of lactate or glucose levels could be used as a surrogate marker of cell number and confluence; flow cytometry or microRNA expression analysis can be used to confirm that the BMSCs have been harvested at the appropriate level of confluence.

Our assessment of BMSC growth showed that confluence affected BMSC proliferation and colony formation, particularly for 100% confluent cells. BMSC PDT fell from 50% to 80% confluence and then increased when they become 100% confluent. The quantity of colonies formed by BMSCs was similar for 50% and 80% confluent cells but was less for 100% confluent cells.

We also found that significant changes in protein expression occurred as confluence increased from 80% to 100% which was manifested by changes in the make-up of BMSC culture supernatant and the expression of surface markers. The supernatant factors that increased steadily included PEDF, which has been reported to be the most potent natural angiogenesis inhibitor and the VEGF/PEDF ratio is closely correlated with the degree of neovascularization[55]. PEDF has also been reported to impair the beneficial effects of MSCs against myocardial infarction injury[56]. We observed an increase in the PEDF/VEGF ratio in 3 of the 4 donors (Figure 2C) which suggests that growing BMSCs to greater than 100% confluence may comprise their pro-angiogenesis properties. Interestingly, bFGF (FGF2), another functionally important BMSC growth factor, was not detected in the culture supernatant, but we were able to detect it in the cytoplasm of BMSCs at a very high level (data not shown). This may, therefore, partially explain the *in vivo* paracrine effects of BMSC, since the trophic factors such as bFGF would be released from the dead cells after infusion[53].

The expression of several BMSC surface markers increased as confluence increased; but many did not increase until the cells reached 100% confluence. It is not known if the increase in expression of these markers are important to the function of BMSCs, but the expression of CD54 (ICAM1) and CD106 (VCAM) have been reported to be induced by IFN-γ or TNF-α[35, 57, 58] and have been associated MSC-mediated immunosuppression[59]. We also found that CD55 was up-regulated in 100% confluent BMSCs. CD55 has previously been detected on BMSCs and can suppress complement activation[60]. Since BMSCs are able to activate the complement system when exposed to human sera[61], it would be interesting to investigate the function CD55 on BMSCs.

In contrast to the changes in protein expression and production, transcription analysis revealed that the most gene expression changes occurred when the confluence increased from 50% to 80%. The genes that were up-regulated in 100% confluent BMSCs compared to 80% confluent cells included Wnt signaling pathway inhibitor genes and angiogenesis inhibitors. This also suggests that 100% confluent BMSCs many have reduced pro-angiogenic activity despite the differences between 80% and 100% BMSCs in the expression of genes and proteins involved with the immune response, there was little difference in the suppression of mixed lymphocyte reactions between 80% and 100% confluent cells. This suggests that it is possible that there is little difference in immunomodulatory functions of BMSCs grown to 80% and 100% confluence.

Since an important regenerative medicine application of BMSCs is bone regeneration, we explored changes in the expression of genes involved with bone formation. We previously demonstrated that *in vivo* bone formation ability of 100% confluent BMSCs was reduced slightly compared with 70% confluent BMSCs, but this difference was donor dependent[62].

In this study we found that Regulation of Ossification (Gene Ontology term, GO: 0030278) was over-represented by 80% confluent BMSCs (p=4.62E-03). The genes contributing to this GO were ANKH, BMPR1B, CSF1, CTNNBIP1, EGR2, IGF1, JUND, PBX1, PRL, RUNX2 and SKI. Interestingly, the expression of these genes was higher in 80% confluent BMSCs compared to 50% confluent cells, but they did not significantly differ between 80% confluence and 100% confluence. However, since the BMSC skeletal stem cell sub-population is quite rare, and our gene expression profiling was performed on bulk RNA, the real signature of SSC may be masked by the majority of the more committed stromal cells. We are now performing single-cell analysis to further characterize the BMSCs, which may provide insightful data on the effects of confluences on SSC.

From the perspective of a GMP-compliant manufacturing facility, harvesting BMSCs at the same degree of confluence for each production lot is essential to provide consistently high quality BMSCs. The practice in our clinical cell therapy laboratory is to harvest BMSCs when they become 80% confluent. Based on the results of this study we will continue this practice. The results of our study suggest that culturing to 100% confluence is detrimental in some respects to BMSCs. BMSCs grown to 50% confluence also differ from 80% confluent cells, but the impact of these differences is less certain. However, harvesting at 50% confluence reduces yields and, as a result, increases the cost of production.

It would be desirable to identify markers that are associated with cell confluence and which could provide a global evaluation of BMSC confluence. Since BMSCs are grown as adherent cell cultures, the cells cannot be sampled to measure surface marker, microRNA or gene expression to determine if they had reached 80% confluence. Culture supernatant, however, can easily be collected and used to assess biomarkers. While the levels of many soluble proteins change during BMSC culture, only when protein levels are normalized for cell number can they be used to predict BMSC confluence. Therefore, supernatant protein levels are of limited use in assessing confluence of these adherent cells. We did, however, find that supernatant glucose and/or lactate values are good indicators of cell density. The GCR and LGR also proved to be highly correlated with cell densities. These measures are markers of BMSC confluence and could be used as an indicator of the optimal harvest time. However, LGR and GCR will depend on culture conditions and culture vessel characteristics, so if changes in lactate and or glucose levels are used, they will have to be validated for each set of culture conditions.

It is often desirable to test intermediate or final products to document their quality. Our results show that the analysis of the expression of surface makers by flow cytometry, particularly CD10 expression, could be used to ensure that BMSCs have been harvested prior to reaching 100% confluence. It may also be possible to use the expression of a panel of microRNA for a more refined measurement to ensure that BMSCs have been harvested when they were greater than 50% confluent but less than 100% confluent.

Of note, these studies involved the culture of bone marrow-derived MSCs in flasks with 20% FBS and the effects of confluence could possibly be different on MSCs grown under other conditions or on MSCs from other tissues. For example, it is not certain if the effects of confluence would be the same on earlier or later passage BMSCs. Confluence may also

have different effects on BMSCs grown in other vessels such as bioreactors, with other culture medium such as serum-free culture medium or on other types of MSCs such as adipose tissue-derived MSCs. In addition, the effects of confluence on the *in vivo* properties of BMSCs could differ from the properties measured *in vitro*. Further studies on the effects of confluence on other types of MSCs, MSCs grown under other conditions and the effects on their *in vivo* properties are warranted.

In conclusion, growing BMSCs to higher confluences increases cell density and yields, but harvesting cells at 100% confluence may not result in cells of the highest quality. The measurement of supernatant lactate and glucose levels can be used to estimate BMSC quantity and confluence and can be used to help ensure consistency in the timing of passage and harvest. Flow cytometry or microRNA expression analysis can be used to confirm that the BMSCs have been harvested at the appropriate confluence.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

BMSC	Bone Marrow Stromal Cell
PEDF	Pigment Epithelium-Derived Factor
VEGF	Vascular Endothelial Growth Factor
PD	Population Doubling
PDT	Population Doubling Time
CFE	Colony Formation Efficiency
aGvHD	acute Graft-versus-Host-Disease
GMP	Good Manufacturing Practice
SOP	Standard Operating Procedures
PI	Propidium Iodide
GCR	Glucose Consumption Rate
LGR	Lactate Generation Rate
MLR	Mixed Lymphocyte Reaction
PCA	Principle Component Analysis

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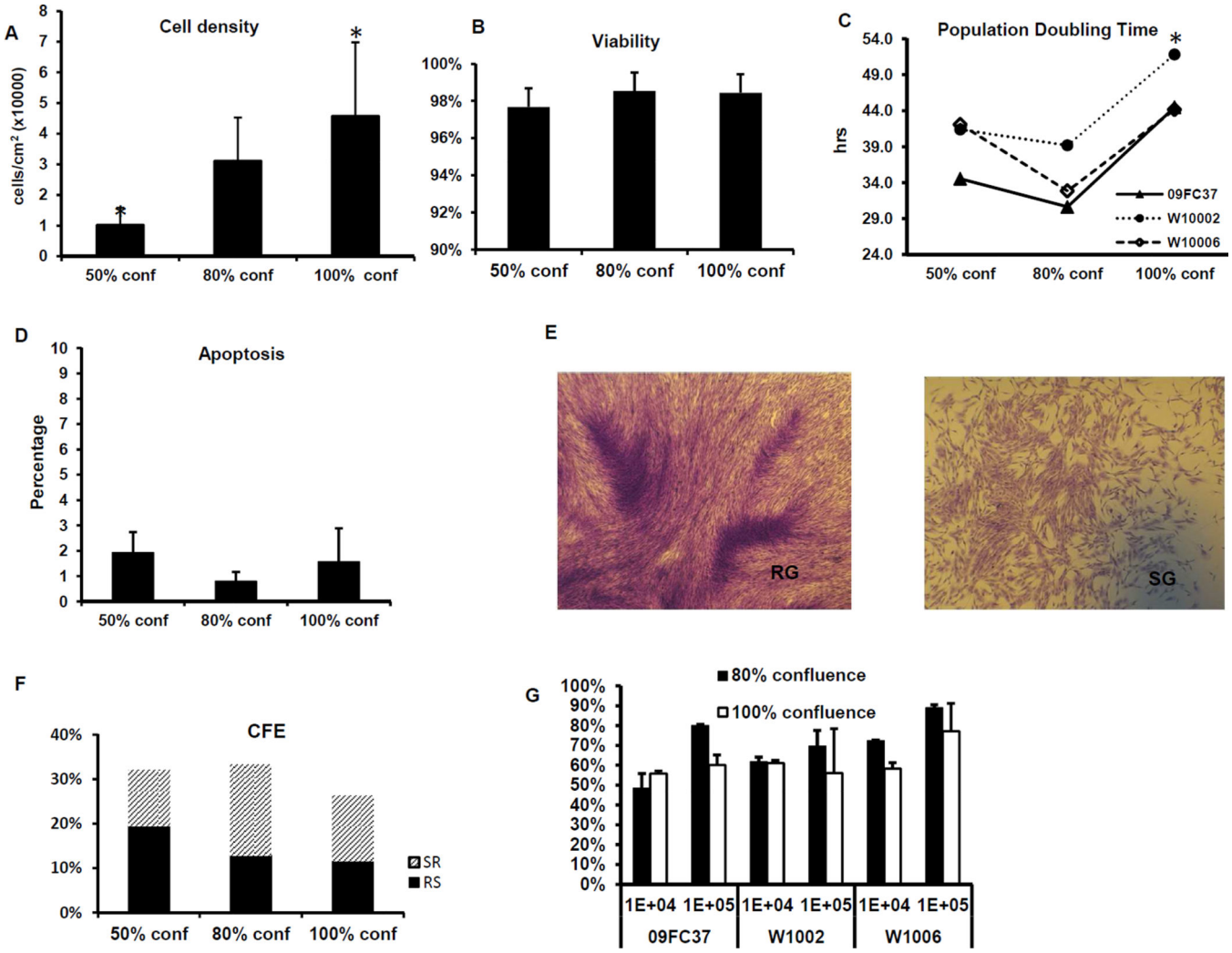

Figure 1. Changes in the phenotypes of 50%, 80% and 100% confluent BMSCs

The BMSCs were cultured to 50%, 80% and 100% confluences, the cell densities per cm^2 culture area were calculated (A) and the viability was determined by Trypan Blue staining (B). In addition, BMSC population doubling time (hours) was calculated (C) and the rate of apoptosis was measured by Annexin V- flow cytometry analysis (D). Representative figures of Rapidly Growing (RG) and Slow Growing (SG) colonies are shown (E). Colony Formation Efficiency (CFE) was counted and the percentage of RG and SG colonies was also shown (F). Immunosuppression of BMSCs of mixed lymphocyte reactions was measured by H^3-thymdine incorporation methods; the percentage of suppression was calculated by normalizing the values to the mixed lymphocyte reaction without BMSCs (G). The * indicates p values <0.05 when compared with 80% confluence and the error bars indicate one standard deviation.

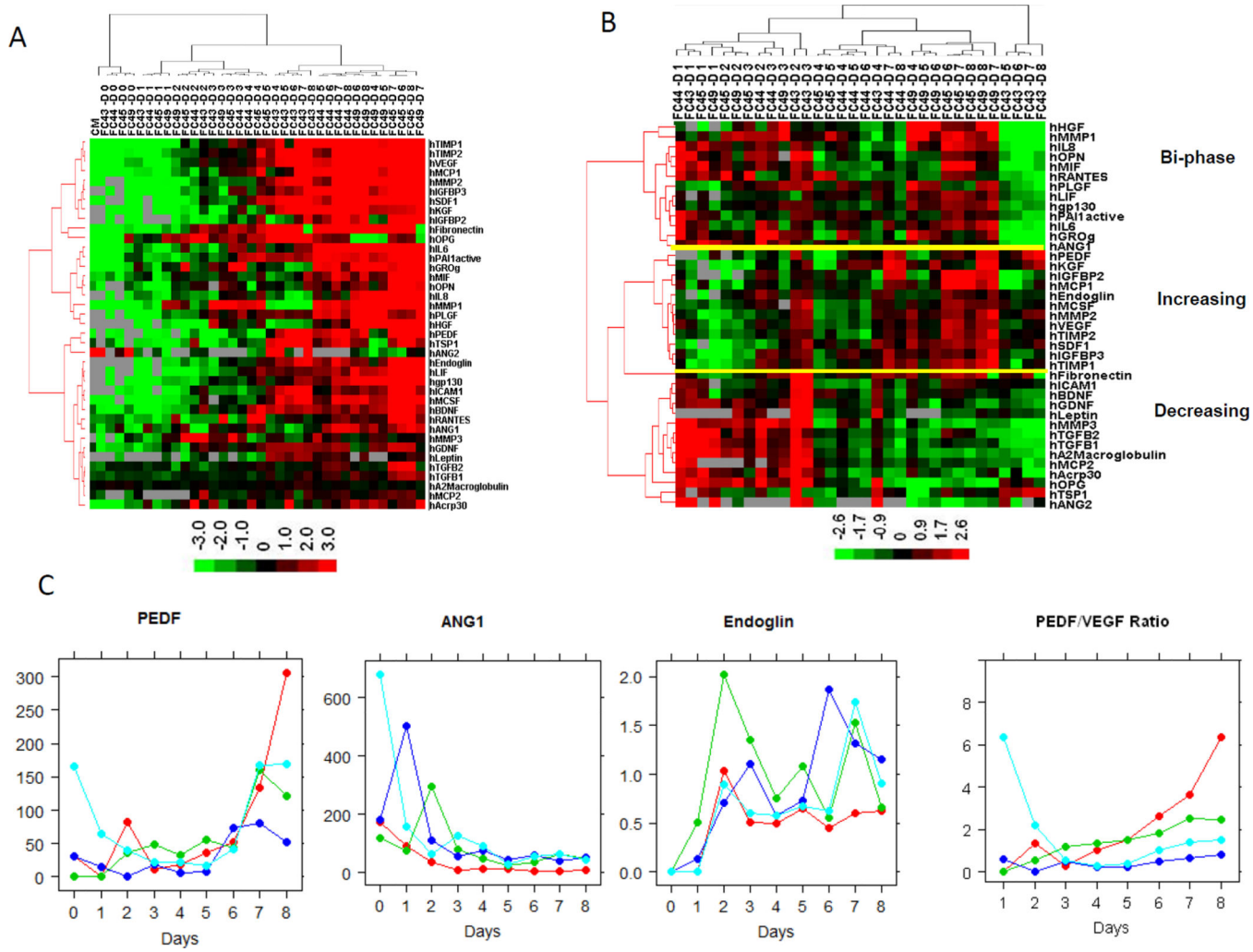

Figure 2. Concentration of proteins in BMSC culture supernatants

The culture supernatants of 4 BMSCs were collected daily for 8 days and the concentrations of proteins were measured by SearghLight. A heat-map of the unsupervised hierarchical clustering analysis of the raw concentrations of 39 proteins were shown in panel A and the protein concentrations normalized by the cell number in panel B. Representative plots of daily normalized supernatant concentrations of PEDF, ANG1 and Endoglin and the ratio of PEDF/VEGF are shown in panel C. In the heat-map, each row stands for one protein, and each column stands for one BMSC supernatant. The donor ID and day of culture were indicated for each sample on the top of heatmap. CM stands for culture medium.

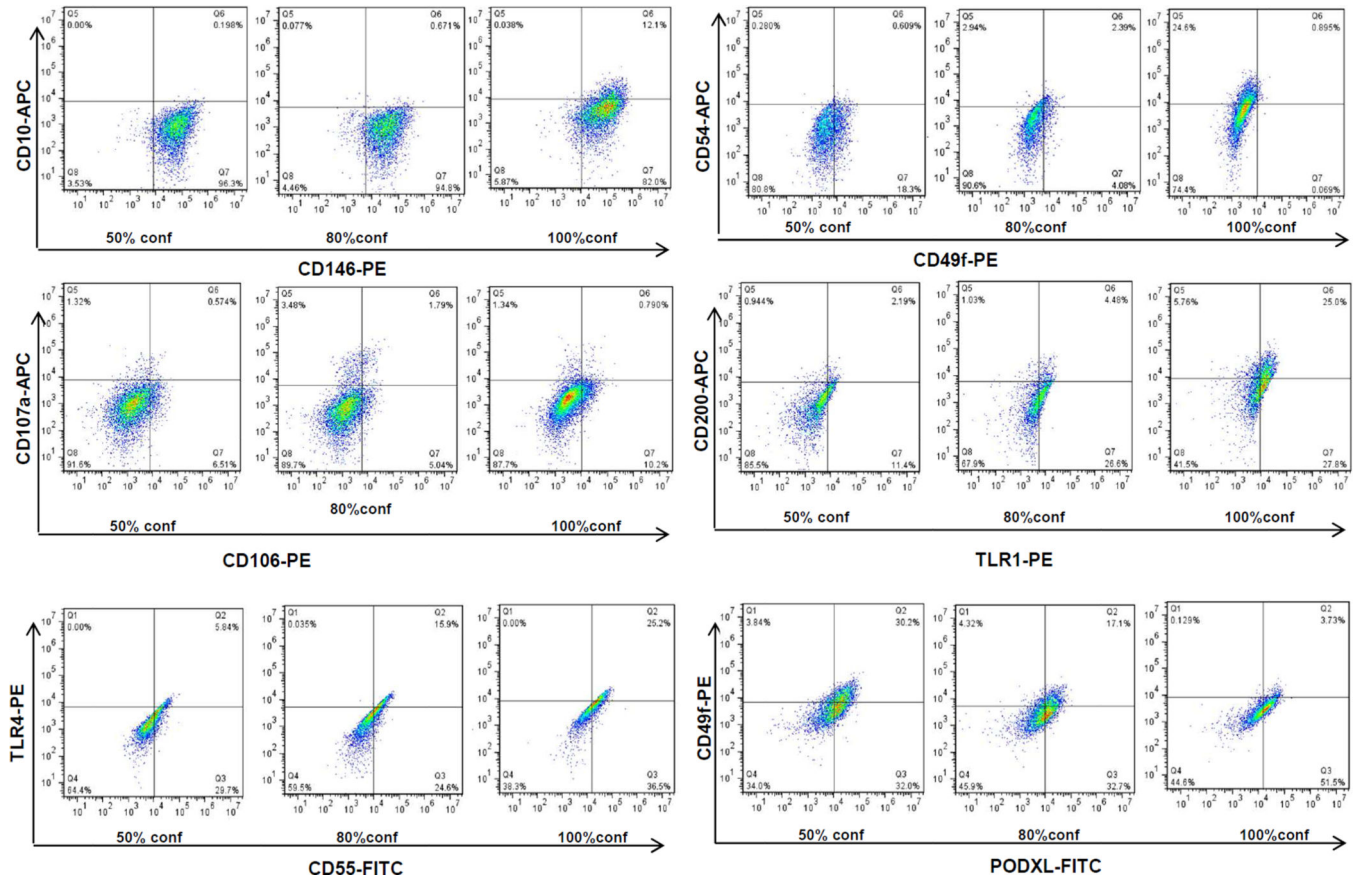

Figure 3. Changes in BMSC surface marker expression at 50%, 80% and 100% confluence

BMSCs from 2 donors were cultured to 50%, 80% and 100% confluence and the expression of surface markers were measured by flow cytometry. Representative plots from one donor were shown. The markers were indicated by x-axis, and y-axis.

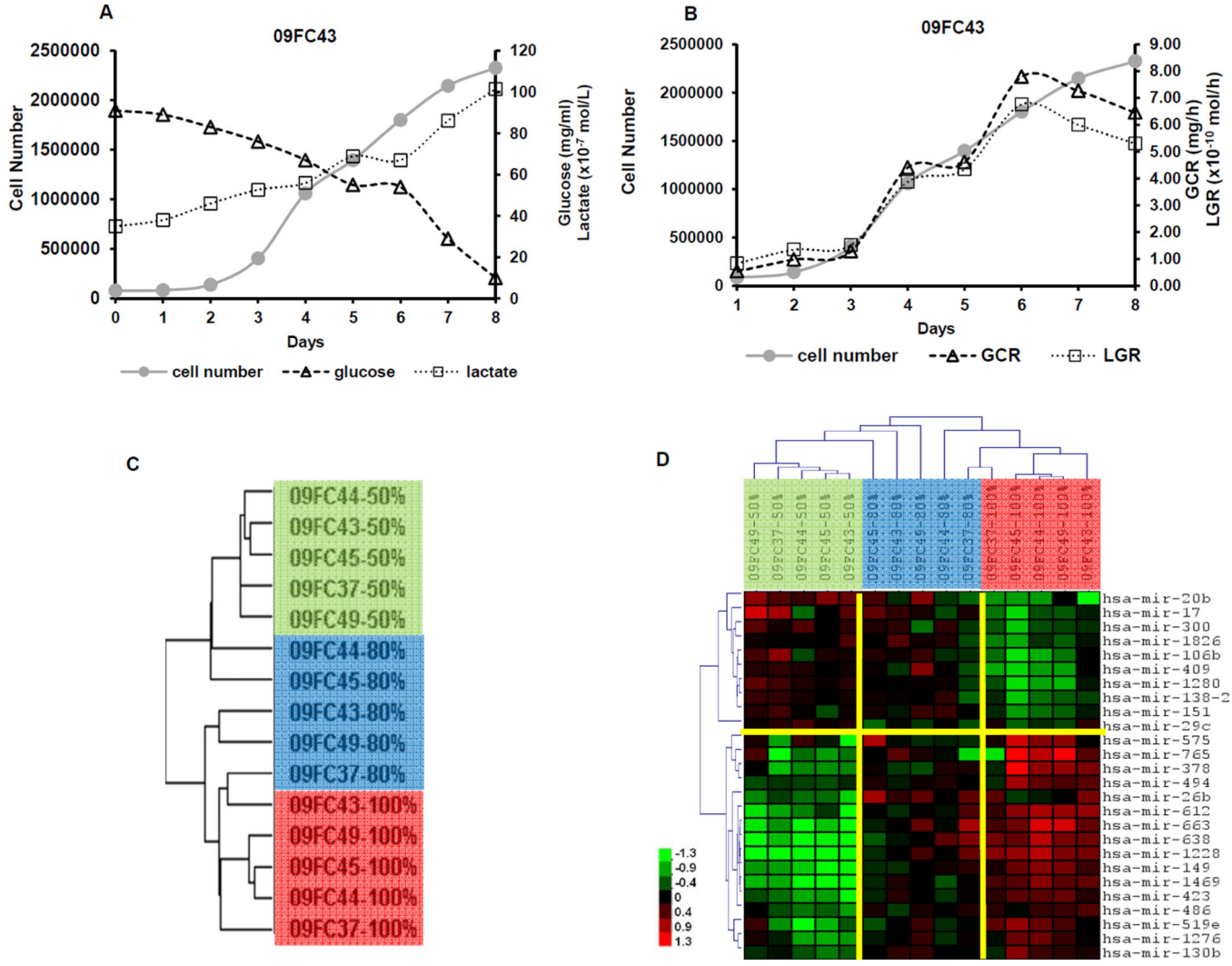

Figure 4. Measuring glucose and lactate in the culture supernatant and microRNA profiling of BMSCs at 50%, 80% and 100% confluence

The BMSC number and culture supernatant concentrations of glucose and lactate were measured daily from 4 donors; the Glucose Consumption Rate (GCR) and Lactate Generation Rate (LGR) were calculated. Representative plot of raw values (A) and calculated GCR and LGR (B) from one donor, 09FC43, are shown.

Unsupervised hierarchical clustering analysis of 166 informative human microRNA separated samples into clusters of 50% confluence (green bar), 80% confluence (blue bar) and 100% confluence (red bar) (C). Twenty-six differentially expressed microRNAs were identified by Two-way ANOVA ($p<0.05$). Hierarchical clustering analysis using the 26 microRNA separated the samples into clusters of 50% confluent (green bar), 80% confluent (blue bar) and 100% confluent BMSCs (red bar). The names of the 26 microRNA are shown on the right of each row (D). Scale Bar indicates the expression level.

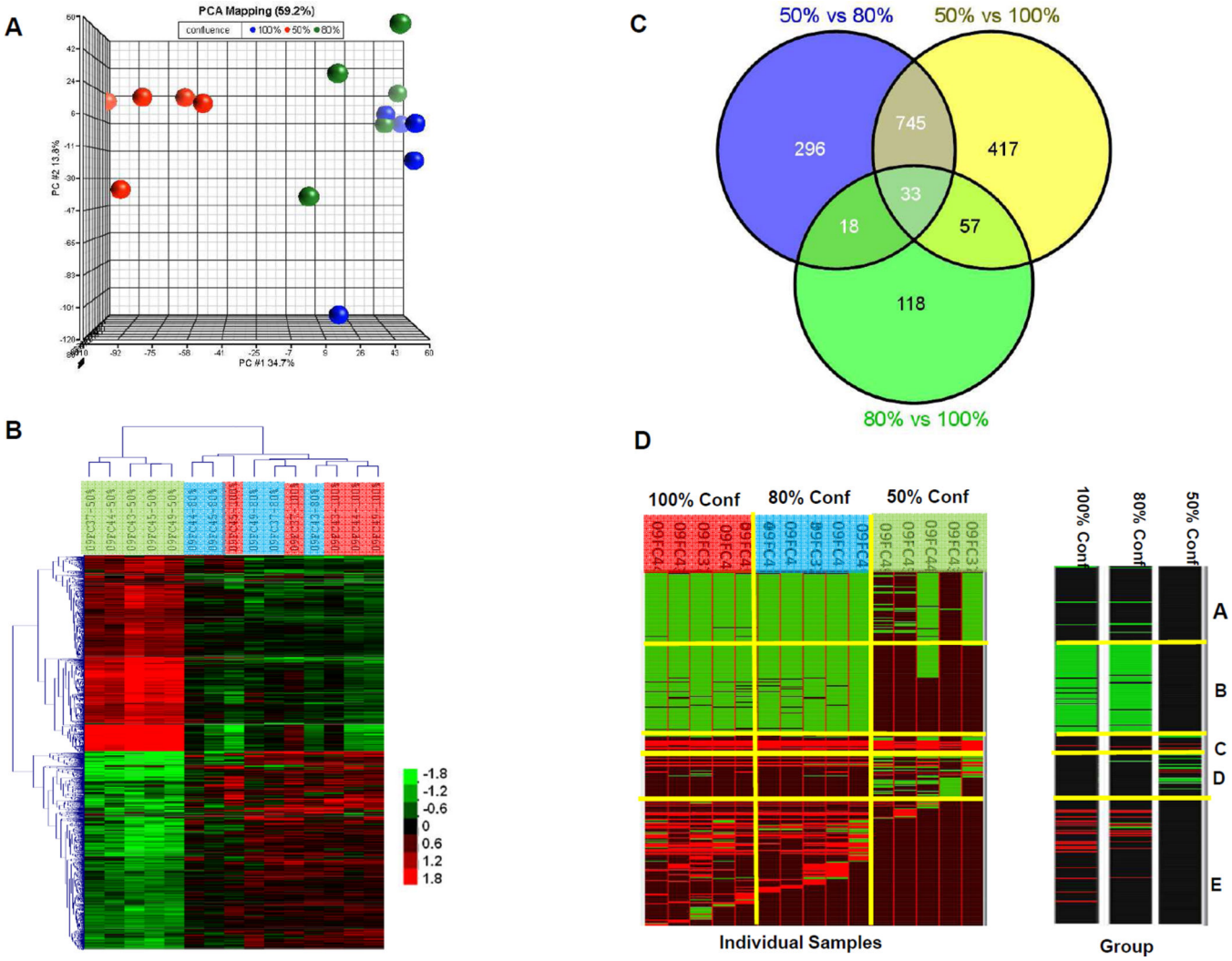

Figure 5. Gene expression profiling of BMSCs at 50%, 80% and 100% confluence

Principal component analysis (PCA) separated the BMSCs into a group with 50% confluent BMSCs (red spheres) and another group with both 80% (green spheres) and 100% confluent BMSCs (blue spheres) (A). Differentially expressed genes ($p<0.001$) were used for hierarchical clustering analysis which separated the samples into a cluster with 50% confluence (green bar) and another cluster with 80% confluence (blue bar) and 100% confluence (red bar). Scale Bar indicates the expression level. (B). The number of genes whose expression differed significantly among groups ($p<0.001$, Fold Change >1.5) are displayed using a Venn Diagram (C). The annotation of differentially expressed genes was conducted using Genomica. Each row stands for one gene set, and each column stands for one individual BMSC sample (D, left) or one of the 3 groups of 50%, 80% and 100% confluent BMSCs (D, right). The green grids indicated “repressed” and red grids indicated “activated”.

Table 1

Correlation between Glucose, Lactate, Glucose Consumption Rate (GCR), Lactate Generation Rate (LGR) and Cell Number

BMSC ID	Glucose		Lactate		GCR		LGR	
	R	p-Value	R	p-Value	R	p-Value	R	p-Value
09FC43	−0.96	4.77E-05	0.95	7.74E-05	0.96	2.11E-04	0.94	4.96E-04
09FC44	−0.89	1.35E-03	0.88	1.55E-03	0.97	5.60E-05	0.98	2.79E-05
09FC45	−0.95	1.14E-04	0.93	2.81E-04	0.97	7.21E-05	0.89	3.26E-03
09FC49	−0.9	1.06E-03	0.88	1.65E-03	0.76	2.84E-02	0.8	1.65E-02

Table 2

MicroRNAs Differentially Expressed Among 50%, 80% and 100% BMSCs

microRNAs	Confluence			P-Value	MSC activities
	50%	80%	100%		
hsa-miR-17	−3.13	−3.41	−4.18	2.9E-02	Down-regulated in aging human MSC, targeting CDKN1A[63] Accelerated 3T3L1 adipocyte differentiation by targeting Rb2/p130[64] promote proliferation of USSC and prevent cell cycle arrest during neuronal lineage by targeting CCND1, E2F1, CDKN1A, PTEN, RB1, RBL1, RBL2, WEE1[65]
hsa-miR-20b	−2.50	−3.10	−4.22	4.4E-03	up-regulated in ES-MSCs and targeting EPAS1[66] expressed in WJ-MSC and down-regulated during WJ-MSC neurogenesis[67]
hsa-miR-106b	−2.40	−2.61	−3.37	1.1E-02	Up-regulated in osteo-differentiated MSCs[68] promote proliferation of USSC and prevent cell cycle arrest during neuronal lineage by targeting CCND1, E2F1, CDKN1A, PTEN, RB1, RBL1, RBL2, WEE1[65]
hsa-miR-29c	0.57	0.08	0.20	1.7E-02	Up-regulated in replicative senescence[69], possibly targeting DNMT3A, DNMT3B[69,70] increased during osteoblastic differentiation of murine cell line and involved in Wnt Signaling Pathway by targeting Sparc[71] reduced expression of extracellular matrix genes[72]
hsa-miR-138	0.92	0.71	0.14	1.4E-02	Down-regulated during osteoblast differentiation of hMSCs; inhibited osteoblast differentiation of hMSCs in vitro and reduced ectopic bone formation in vivo by targeting FAK[73] Down-regulated in osteo-differentiated MSCs[68] Up-regulated in osteo-differentiated MSC[49] Down-regulated during adipogenic differentiation of human ASC; inhibited adipogenesis by targeting EID-1[9] up-regulated during WJ-MSC neurogenesis[67]
hsa-miR-26b	−0.39	0.82	0.49	4.3E-03	up-regulated during osteoblast differentiation of hMSCs[73] up-regulated during chondrogeneic differentiation of human BMSC, possibly targeting *COL4A1, COL2A1, and COL6A1*[74] up-regulated expression in wound tissue at the stage of active granulation formation[75] Detected in human ES derived MSC[76]
hsa-miR-130b	−0.62	−0.07	0.08	2.1E-02	Up-regulated in osteo-differentiated MSCs[68] and chondro-differentiated MSCs[74] Detected in human ES derived MSC[76] up-regulated expression in wound tissue at the stage of active granulation formation[75]
hsa-miR-494	−0.23	0.20	0.59	4.9E-03	Differentially expressed in osteo-differentiated MSC with Donor variance[68]
hsa-miR-663	−1.40	0.36	0.93	1.4E-04	Differentially expressed in osteo-differentiated MSC with Donor variance[68] Exclusively expressed in chondroblast derived from human MSC[49]

microRNAs	Confluence			P-Value	MSC activities
	50%	80%	100%		
					2 fold higher in MV than ES-MSC[76]
hsa-miR-638	−1.67	0.05	0.66	1.1E-05	Exclusively expressed in chondroblast derived from human MSC[49] down-regulated in osteo-differentiated MSC[49] 2 fold higher in MV than MSC[76]
hsa-miR-486	−0.37	0.20	0.59	2.8E-03	Up-regulated in aging human ASCs, induces a premature senescence-like phenotype and inhibits proliferation; inhibits adipogenic and osteogenic differentiation by targeting SIRT1[77]

USSC: unrestricted somatic stem cells from human cord blood. **ES-MSC**: human ES-derived MSC. **WJ-MSC**: Wharton's jelly matrix-derived mesenchymal stem cell. **ASC**: Adipose-tissue derived stem cell. **MV**: microvesicles