ORIGINAL ARTICLE

Comparative Evaluation of Mouse Bone Marrow Mesenchymal Stromal Cells Characteristics Cultured in Two Different Supplemented Media

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ABSTRACT

Introduction: Preclinical studies on mesenchymal stromal cells (MSC) have allowed the cells to be considered as a promising candidate for cellular therapy. In recent years, conflicting data have been reported regarding various aspects of their characteristics, development and differentiation potential, which may be due to arrange of factors. Among the factors worth investigating is the culture medium formulation. **Methods:** Here we have made a comparative characterization of mouse bone marrow mesenchymal stromal cells (mBM-MSC) that were cultured using two common supplements, MesenCult™ Stimulatory Supplement (MSS) and fetal bovine serum (FBS), under the same experimental conditions at different passages. Clonogenic potential, cumulative population doubling level (CPDL), population doubling time (PDT), immunophenotyping, differentiation, immunosuppression potentials and chromosome analysis of early and late passages mBM-MSC were assessed. **Results:** Our findings showed that the CPDL, immunophenotype and immunosuppression potential of mBM-MSC were similar. However, variations were seen in their clonogenicity, population doubling time and differentiation efficacy whereby all of these were enhanced in DMSS. These observations suggest that their genetic make-up may be affected by both supplements upon prolonged culture. Interestingly, this conjecture was supported when chromosomal analysis revealed genetic instability of mBM-MSCs cultured in both supplements. **Conclusion:** In conclusion, culture medium formulation was shown to cause variations and spontaneous transformation in mBM-MSCs raising concerns on the usage of late passages mBM-MSCs in fundamental and preclinical downstream experiments.

Keywords: Mesenchymal stromal cell, Characterization, Mouse bone marrow

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INTRODUCTION

Mesenchymal stromal cells (MSCs) are a unique population of adult stem cells, possessing not only the basic characteristics of stem cells, i.e., self-renewal and multi-lineage differentiation potential, but also hematopoietic and immunomodulatory function (1,2). It was previously reported that MSCs were able to exert immunosuppression on a variety of immune cells including T cells, B cells and NK cells via the production of nitric oxide and the interactions with cytokines released by MSCs such as IL-10, TGF-β, IDO, and PGE2 (3). Besides, MSCs are robust and easily isolated from various sources such as bone marrow, adipose tissue, umbilical cord, and amniotic fluid making them an ideal candidate for cellular therapy and regenerative medicine (4–6). Recent findings on the immunosuppressive properties of MSCs have increased the needs for consistent supply of MSC for experimental research and clinical trials (7,8). The expansion of undifferentiated MSCs that exhibit stemness and differentiation ability remained steadfastly important in our quest to further understand and utilize these stem cells to their fullest potential. Being so, one of the more popular sources is murine MSC, which has been extensively used for preclinical studies in this field.

Murine MSCs are commonly obtained from the bone marrow aspirate of the femur and tibia and taken as a

standard for comparison of MSCs from other sources. However, in the lack of a standardized isolation and expansion method, variations in the cultured MSCs exist within research groups making it hard to correlate all the findings (9). In general, MSCs isolation takes advantage of its plastic adherence characteristic, which is not always the best method as it will result in heterogenous population. Furthermore, identifying MSCs based on their surface markers remains quite impossible due to the interspecies differences of the surface markers expression (10,11). Therefore, various factors have to be taken into consideration for MSCs characterization such as the species and tissue-specific surface markers and also the functional analysis which is well accepted verification that is not dependent on species or tissue type.

MSCs have been routinely isolated and cultured in different types of complete media, with fetal bovine serum (FBS) supplemented media being among the common ones (12,13). FBS is a crucial component in cell culture media as they provide all the essential nutrients, growth factors and chemokines that are vital for cell growth and proliferation. However, there are some concerns regarding the efficacy and safety of FBS-based culture as they can trigger adverse immune responses as previously reported (14). Therefore, in this study we tested and compared MesenCult™ Stimulatory Supplement (MSS) medium against the commonly used FBS-supplemented medium. MSS-supplemented medium is one of the common optimized-media used for the isolation and culture of bone-marrow derived MSCs (15,16). Likewise with FBS, MSS is an important source for essential nutrients and growth factors specially optimized for the isolation and expansion of MSCs. However, it is still unclear how these culture conditions influence the phenotype and function of the expanded MSCs. Hence, in this study we tested these two media to compare the population doubling time, phenotypic and functional characteristics of the isolated and expanded mouse bone marrow MSCs (mBM-MSCs).

MATERIALS AND METHODS

Isolation and culturing of mBM-MSCs

Ethical clearance (UPM/IACUC/AUP-R079/2014) from the Institutional Animal Care and Use Committee (IACUC) of Universiti Putra Malaysia was obtained on 9th March 2015, prior to the commencement of the experiments. Isolation of the mBM-MSCs was carried out on the femur and tibia of 8 weeks old C57BL/6 mice according to the protocol by Soleimani and Nadri with some modifications (12). Bone marrow aspirate was flushed out of the bones by using a 27G needle (Terumo, Japan). Yield and viability was assessed using Trypan blue exclusion assay. The cells were then seeded into T25 flask at 1 x 10^6 cells/cm^2 seeding density in two different complete media. DMEM high glucose with L-glutamine (Biowest, France) and 1% antibiotic/antimycotic (Biowest, France) supplemented with either 15% of MesenCultTM Stimulatory Supplement (Stemcell Technologies, Canada) (DMSS) or 15% fetal bovine serum (Biowest, France) (DFBS) were used to maintain the mBM-MSCs in two independent but parallel cultures. In order to minimize variation, media and supplements used were from the same batch lot. Media change was carried out every two days to replenish the used media. Sub-culture was performed once the cells reached 70-80% confluency and maintained in their respective media for further experimental purposes. Seeding density for the expansion of the MSCs were maintained at 5 x 10^3 cells/cm^2. The cells were passaged until passage 40 (late passage). Cells were grown at 37°C in an atmosphere of 5% CO_2, and relative humidity of above 90%. Three biological replicates were used for the study.

Colony forming unit-fibroblasts (CFU-F) assay

The clonogenic potential of the isolated bone marrow cells were assessed by colony forming unit-fibroblasts (CFU-F) assay according to a protocol by Pochampally (2008) (17). The cells were plated in 6-well tissue culture plates at passage 0 in triplicate cultures at seeding density of 1x10^6 cells/well using respective complete media. The media were replenished every two days. The CFU-F was assessed after 14 days of culture. Cells were washed twice with PBS pH 7.2 (Gibco, USA) before fixing with methanol (Merck, Germany) for 10 minutes. The cells were stained with 0.5% crystal violet (Merck, Germany) prepared in methanol for 30 minutes at room temperature. The cells were then rinsed with PBS three times and once with distilled water and air dried. The colonies were viewed and scored under a compound light microscope (Olympus, Japan). Colonies with at least 50 cells were scored. Graph were plotted and statistical analysis using Student's t-test were performed using GraphPad Prism 5.

Cumulative population doubling level and doubling time

Cumulative population doubling level (CPDL) is the total number of times cells in a given population doubled during in vitro culture. Population doubling time (PDT) is the time needed for the isolated mBM-MSCs to proliferate and double in numbers. Cells were seeded at a seeding density of 5 x 10^3 cells/cm^2 and were passage upon reaching 80%. During the continuous passages, the number of mBM-MSCs at both seeding and harvesting were determined to calculate the CPDL and PDT (18). Cells were sub-cultured every 96 hours for the CPDL and PDT calculations. Cells were counted using Trypan blue exclusion method, where stained blue cells were scored as non-viable, while unstained cells were scored as viable cells using hemocytometer. The CPDL and PDT were then calculated using the following formula:

PDL = ln(Nf/Ni) ln2

CPDL = PDL + X

PDT = CT/PDL,

where, Ni is the initial cell number, Nf is the harvest

cell number, ln is the natural log, X is the PDL of the previous passage and CT is cell culture time (hours) for each passage. Graph were plotted and statistical analysis using Student's t-test were performed using GraphPad Prism 5.

Immunophenotyping

An important part of MSCs characterization is the expression of their surface markers. Cells from passage onwards were immunophenotyped at an interval of every five passage up to passage 40. Immunophenotyping was carried out with selected conjugated antibodies against mouse MSCs markers as follows; $CD44^+$, $Sca\text{-}1^+$, $CD106^+$, $CD11b^-$, $CD45^-$ and their respective isotype controls (BD Biosciences, USA). Briefly, cells were harvested and washed with PBS pH7.2 before being distributed into their respective facs tubes (BD Biosciences, USA) at approximately $5x10^5$ cells per tube. Cells were washed once with PBS and centrifuged at 400 x g for 5 minutes. The supernatant was decanted and 1 µL of the fluorochrome conjugated antibodies were added into respective tubes. The cells were then incubated for 30 minutes at 4°C in dark. After that, the cells were rinsed with PBS and centrifuged again. The supernatant was decanted and the cells were resuspended in 500 µL of PBS prior to flow cytometry analysis using FACS Canto II (BD Biosciences, USA). Graph were plotted and Student's t-test statistical analysis were performed using GraphPad Prism 5.

Differentiation Assay

Another crucial assay to determine the stemness of MSCs is the differentiation assay. In this study, MSCs were induced to differentiate into the adipocytes, osteoblast and chondrocytes using their respective induction media. The differentiation assays were carried out every five passages up to passage 40.

Adipogenesis Assay

For the adipogenic differentiation assay, mBM-MSCs were cultured using StemPro® Adipogenesis Differentiation Kit (Thermo Fisher Scientific, USA). Briefly, cells were seeded in 12-well tissue culture plate (TPP, Switzerland) with seeding density of $1x10^4$ cells/cm2 in respective complete media and incubated at 37°C with 5% CO2. After the cells reached 80% confluency, the spent media were replaced with the complete adipogenesis differentiation medium and incubated for another 21 days. The differentiation medium was changed every 3 days. After 21 days under the differentiating condition, the spent medium was removed from the culture plate and the cells were rinsed twice with 500 µL of PBS. Then, 10% formalin solution (Sigma-Aldrich, USA) was added and the cells were fixed for 30 minutes. After fixation, the cells were rinsed twice with ultrapure water. Next, 1 mL of 60% isopropanol (Merck, Germany) was added into the wells and incubated for 5 minutes at room temperature. After removing the isopropanol, 1 mL of working solution of Oil Red O stain (Merck, Germany) was added and incubated for 5 minutes. Then, the Oil Red O staining solution was removed, and the wells were rinsed with distilled water. After that, the cells were counter stained with 1 mL of hematoxylin solution (Sigma-Aldrich, USA) for 2 minutes at room temperature. The hematoxylin solution was then removed and the cells were rinsed with 1 mL of tap water for 3 times. Before viewing under microscope for red colored lipid droplets, 1 mL of tap water was added into the well. Images were captured for further qualitative analysis.

Osteogenesis Assay

For the osteogenic differentiation assay, mBM-MSCs were cultured using StemPro® Osteogenesis Differentiation Kit (Thermo Fisher Scientific, USA). Briefly, cells were seeded in 12-well tissue culture plate with the seeding density of $1x10^4$ cells/cm^2 in respective complete media and incubated at 37°C with 5% CO2. After the cells reached 80% confluency, the spent media were replaced with the complete osteogenesis differentiation medium and incubated for another 21 days. The differentiation medium was changed every three days. After 21 days under the differentiating condition, the medium was removed from the culture plate and the cells were rinsed with 500 µL of PBS for two times. Then, 10% formalin solution (Sigma-Aldrich, USA) was added and the cells were fixed for 30 minutes. After fixation, the cells were rinsed twice with distilled water prior to staining with 2% Alizarin Red solution (pH 4.2) (Merck, Germany) for 45 minutes. The cells were then rinsed three times with distilled water and then visualized under light microscope. Images were captured for further qualitative analysis.

Chondrogenesis Assay

For the chondrogenic differentiation assay, mBM-MSCs were cultured using StemPro® Chondrogenesis Differentiation Kit (Thermo Fisher Scientific, USA). Briefly, 5 µL droplets of cells in high density of $1.6 x10^7$ cells/mL were seeded in 12-well plates to form micromass culture. After cultivating micromass culture for 2 hours under high humidity conditions, chondrogenesis differentiation medium was supplemented to the culture and incubated at 37°C with 5% CO_2. The differentiation medium was changed every 3 days. After 21 days under the differentiating condition, the medium was removed from the culture plate and rinsed with 500 µL of PBS once. Then, 10% formalin solution (Sigma-Aldrich, USA) was added and the cells were fixed for 30 minutes. After fixation, the cells were rinsed with 500 µL of PBS and stained with Alcian Blue solution (Merck, Germany) for 30 minutes. Then, the wells were rinsed three times with 0.1 N HCl (Merck, Germany) before adding distilled water to neutralize the acidity and viewed under the microscope. Images were captured for further qualitative analysis.

Immunosuppressive activity of mBM-MSCs on human peripheral blood mononuclear cells (PBMC)

Human ethical approval was obtained on 8th August 2017, prior to this study (JKEUPM (FPSK-P112) 2017). The immunosuppression was conducted based on the modified protocol from Ren et al. (41). After obtaining written consent from a healthy donor, 4 mL of blood was withdrawn by a trained phlebotomist into a sterile EDTA blood tube (BD, USA). Human peripheral blood mononuclear cells (PBMC) were isolated using Ficoll-Paque solution (Sigma-Aldrich, USA) according to the manufacturer's protocol. Briefly, 4 mL of blood were diluted with 4 mL of PBS pH7.2 and mixed well before being layered onto the Ficoll-Paque solution at a ratio of 2:1 in a 15 mL conical tube (BD Falcon, USA). The solution was then centrifuged at 400 x g for 30 minutes at 20°C. The mononuclear cells layer, which lies in the interphase between the plasma layer and the Ficoll-Paque layer was then transferred into a sterile 15 ml conical tube. The mononuclear cells were rinsed twice with sterile PBS and centrifuged before the addition of the RPMI 1640 (Gibco, USA) complete medium supplemented with 10% FBS and 1% β-mercaptoethanol (Sigma-Aldrich, USA). The isolated mononuclear cells were stained with CFSE (Affymetrix, USA) according to the manufacturer's protocol and co-cultured with mBM-MSCs at different ratio of mBM-MSCs:PBMC of 1:5, 1:10 and 1:25, respectively for the immunosuppresion assay. For mitogenic stimulation, the cells were stimulated with 5 µg/mL phytohemagglutinin (Sigma-Aldrich, USA). The co-cultured cells were maintained in RPMI 1640 complete medium in 37°C incubator for 72 hours. Flow cytometry analysis was carried out to determine the suppression rate of PBMC by mBM-MSCs. Graph was plotted and statistical analysis using two-way ANOVA and Student's t-test was performed using GraphPad Prism 5.

Chromosomal analysis

In order to assess the chromosomal stability of the mBM-MSCs, we performed chromosomal analysis according to previous protocol by Sreejit, Dilip, and Verma (2012) mBM-MSCs from passage 5 and 40 in both DMSS and DFBS media were subjected to the chromosomal analysis (19). Briefly, upon obtaining 70-80% confluency, the BM-MSCs were treated with the culture media containing 10 µl/mL Colchemide (Sigma-Aldrich, USA) for 4 hours before harvesting by trypsinization. Cells were then treated with hypotonic solution of 0.56% KCl (Sigma-Aldrich, USA) prepared in ultrapure water for 15 minutes. Subsequently, the cells were fixed using freshly prepared and chilled Carnoy's fixation solution (methanol: acetic acid at 3:1 ratio). Slides were prepared using the standard air-drying method and stained with Giemsa. Twenty metaphase spreads were analyzed and the chromosome numbers were counted. The average chromosome numbers of the cells were plotted in a bar chart and Student's t-test statistical analysis were performed using GraphPad Prism 5.

RESULTS

Isolation and expansion of mBM-MSCs

Three independent mBM-MSCs cell lines were successfully propagated in two different complete media; DMSS and DFBS. Non-adherent cells were removed by replacing media during the first 3 days of isolation. Morphology of the isolated mBM-MSCs was observed under phase-contrast light microscope (Figure 1). Heterogenous population was observed in the early passages of mBM-MSCs (Figure 1A and 1B) and gradually became more homogenous from passage 5 onwards (Figure 1C and 1D). Homogenous mBM-MSCs that exhibited spindle-shaped fibroblastic morphology were continuously observed till passage 40 in DMSS culture (Figure 1E), however the cells lost their spindle-shaped fibroblastic morphology in DFBS passage 40 culture and acquired an epithelial-like morphology (Figure 1F).

CFU-F Assay

To assess the clonogenicity of the mBM-MSCs, CFU-F assay was carried out on passage 0 of the isolated cells. The colonies formed in DMSS medium were generally

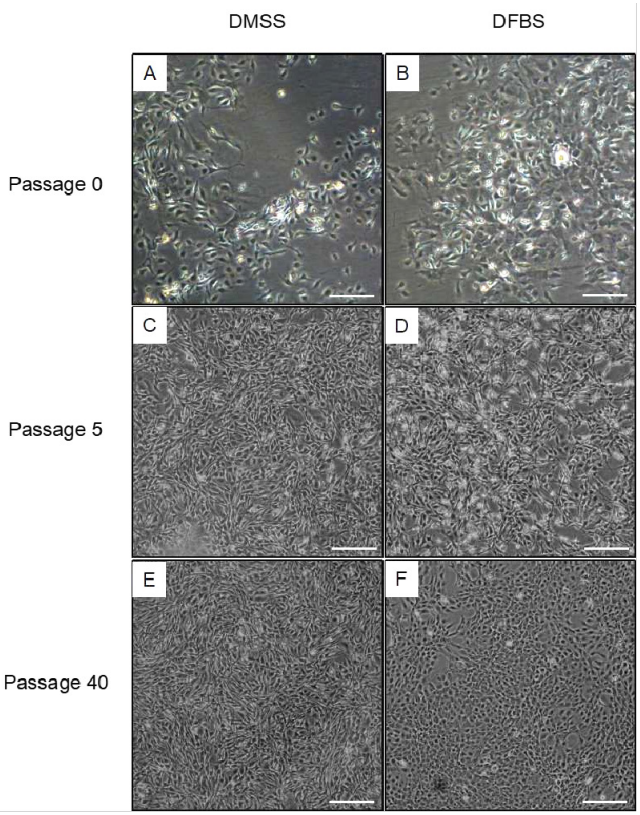

Figure 1: Morphological characterization of mBM-MSCs cultured in DMSS and DFBS media. Heterogeneous culture was observed in passage 0 for both media (A) DMSS and (B)DFBS. A more homogenous population of cells with MSCs prominent spindle shape fibroblastic morphology was observed from passage 5 onwards in both culture media (C) DMSS and (D) DFBS. Similar MSCs morphology was also observed in DMSS culture medium even in late passage (passage 40) as shown in (E). However, the cells acquired an epithelial-like morphology in DFBS (F). Scale bar indicates 100 µm.

larger with more densely packed cells forming these colonies (Figure 2A) compared to the ones in the DFBS medium (Figure 2B). On average, cells cultured in DMSS medium showed significantly higher number of colonies formed at approximately 21 colonies per well compared to only 7 colonies per well for cells grown in DFBS medium (Figure 2C).

Cumulative population doubling level and doubling time

In order to determine the growth kinetic of the isolated mBM-MSCs, cumulative population doubling level (CPDL) and population doubling time (PDT) was carried out at selected passages from early to late passages (Figure 3). There was no significant difference between the mBM-MSCs cultured in DMSS and DFBS for their CPDL across the passages. CPDL for cells in both media showed a steady linear increase from 1.84 (DMSS) and 1.42 (DFBS) to 41.7 (DMSS) and 36.29 (DFBS) for passage 1 to 40, respectively. This gave an indication that the cells were actively proliferating even up to passage 40.

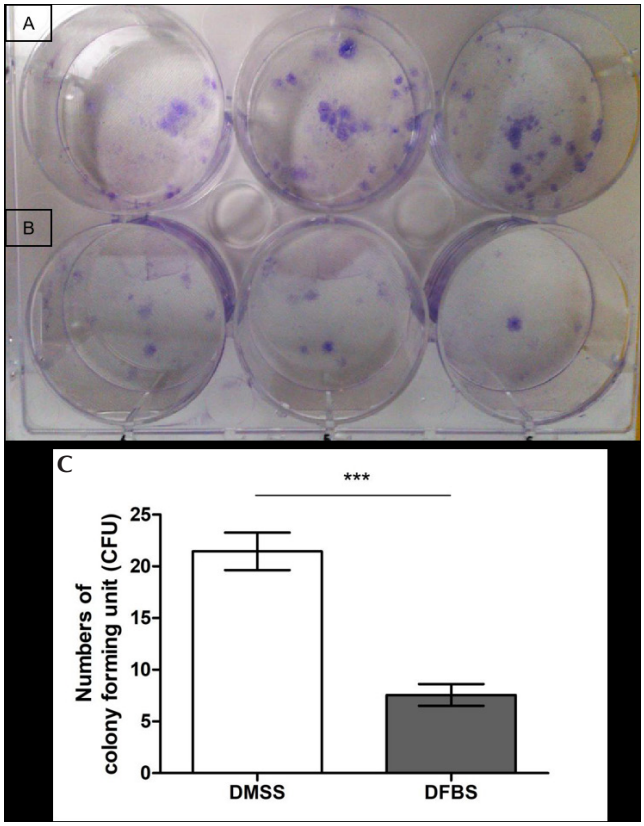

Figure 2: Colony forming unit-fibroblasts (CFU-F) assay. Row (A) were the mBM-MSCs cultured in DMSS medium, while Row (B) were the mBM-MSCs in DFBS medium. Cells were seeded at 1x10^6 cells/well in respective media and cultured for 14 days prior to CFU-F assay. Results shown are mean ± SEM of CFU-F of mBM-MSCs in both media from 3 independent experiments, (C). The number of colonies in DMSS medium is significantly higher compared to those in DFBS medium (p-value ≤ 0.001). Size of the colonies in DMSS medium is also generally larger compared to the DFBS medium.

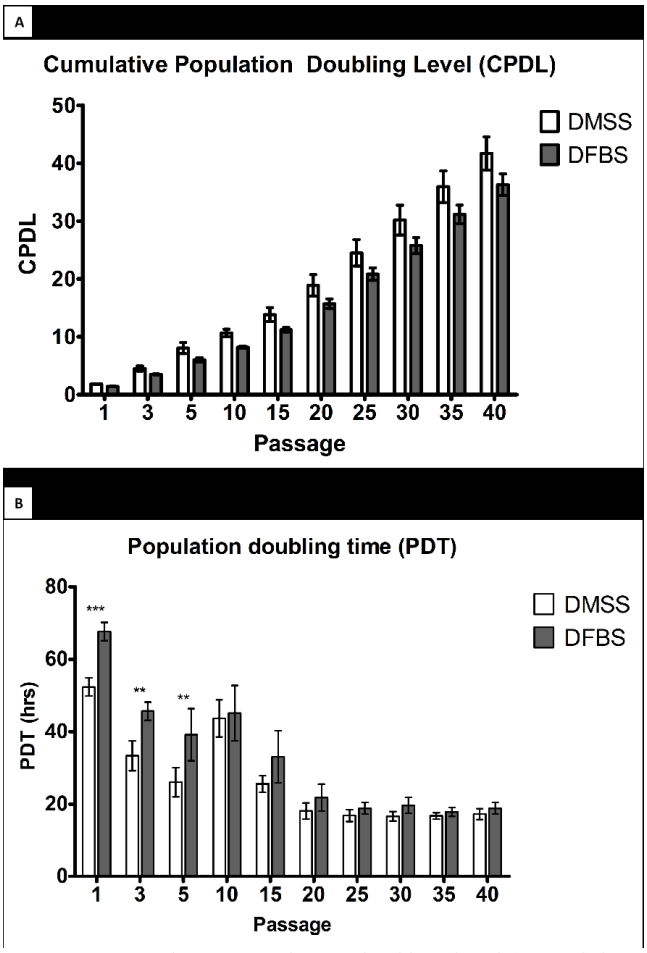

Figure 3: Cumulative population doubling level (A) and doubling time (B) of mBM-MSCs cultured in DMSS and DFBS media. Results shown are mean ± SEM from 3 independent experiments. Generally, CPDL showed a gradual increase across passages but there was no significant difference when compared between DMSS and DFBS. PDT for both sets of mBMMSCs decreases across passage with the exception at passage 10 and remained fairly consistent from passage 20 onwards. Comparing between the mBM-MSCs in the two media, cells in DMSS medium has significantly lower PDT at passage 1, 3 and 5, showing a better growth rate at the earlier passages as compared to cells in DFBS medium.

PDT at passage 1, 3 and 5 showed significant difference between the mBM-MSCs cultured in DMSS and DFBS media. The average PDT at passage 1, 3 and 5 for cells grown in DMSS medium was around 52 hours, 33 hours and 26 hours respectively. Meanwhile PDT for cells cultured in DFBS medium for the same passages was approximately 68 hours, 46 hours and 39 hours, respectively. This showed a gradual decrease in the PDT for both media in the early passages. However, there is an increase in PDT at passage 10 in both media to approximately 43 hours and 45 hours for cells in DMSS and DFBS, respectively. The PDT then decreased further and remained relatively constant throughout the late passages (passage 30 to 40) at 17 hours and 19 hours for DMSS and DFBS media, respectively.

Immunophenotyping

Immunophenotyping was carried out to determine the expression of MSCs surface markers of the isolated mBM-MSCs (Figure 4). The expression of $CD44^+$ (homing cell adhesion molecule) on the cells was consistently high, above 90% throughout the passages (passage 5 to 40) in both media cultures. The expression of $Sca\text{-}1^+$ (stem cell antigen-1) was also consistently high especially in DFBS medium, above 80%, throughout the passages. The expression of $Sca\text{-}1^+$ in cells cultured in DMSS medium, however fluctuated and dipped below 80% from passage 20 upto passage 40. The expression of $CD106^+$ (vascular cell adhesion molecule) was inconsistent throughout the passages in both media cultures. But generally, the expression of $CD106^+$ was higher in cells cultured in DMSS medium. The expression for the MSC negative markers, $CD11b^-$ (Integrin alpha M) and $CD45^-$ (Protein tyrosine phosphatase, receptor type C) were generally low, i.e., below 5% throughout the passages (passage 5 to 40), suggesting that the cultures in both media maintained their MSCs phenotype even up to passage 40. There were no significant differences found between the surface markers across the passages and also between the media.

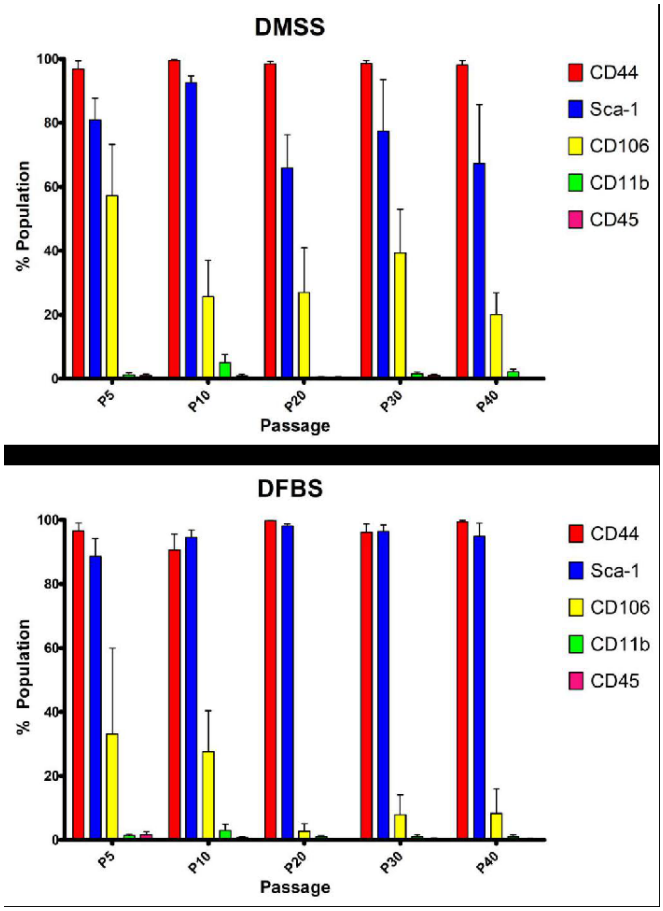

Figure 4: Immunophenotyping of mBM-MSCs. Cells cultured in DMSS and DFBS media both showed strong positive for CD44 and Sca-1 markers. CD106 expression fluctuated but was generally higher in cells cultured in DMSS medium. All cells showed negative expression for CD11b and CD45. Values represent mean ± SEM from 3 independent experiments. No significant differences found when compared across the passages and between media.

Differentiation ability

Differentiation assay was carried out to differentiate the cells into adipocytes, osteoblasts and chondrocytes to confirm that the propagated mBM-MSCs maintained their MSCs characteristics. Cells cultured in both media were able to differentiate under the specific induction media to form adipocytes, osteoblasts and chondrocytes respectively. Cells were able to differentiate starting from passage 5 onwards up to passage 40. They did not lose the ability to differentiate across prolonged passage. This was shown in Figure 5 as the Oil Red O stained the lipid vacuoles from adipocytes (Figure 5B-C), Alizarin Red stained the calcium deposits produced by osteoblasts(Figure 5E-F) and Alcian Blue stained the proteoglycan produced by chondrocytes (Figure 5H-I) and their respective controls which did not pick up any staining (Figure 5A, D and G). Although this is a qualitative assay, it was observed that the cells cultured in DMSS have stronger staining in both adipogenic and osteogenic differentiation assays as compared to cells cultured in DFBS. Chondrogenic differentiation assay did not show any difference as both sets of cells, either in DMSS or DFBS showed similar differentiation ability. These differentiation assays suggest that DMSS medium give greater differentiation efficacy compared to DFBS medium to the mBM-MSCs.

Immunosuppression activity

The immunosuppression assay showed that the immunosuppressive activity of passage 5 mBM-MSCs was significantly higher than that of passage 40 for DMSS at 1:5 ratio (p-value ≤ 0.01) and DFBS at 1:25ratio (p-value ≤ 0.05) (Figure 6). The highest immunosuppression was observed at passage 5, where 63% and 75% of PBMC were suppressed by mBM-MSCs cultured in DMSS and DFBS, respectively at mBM-MSCs to PBMC ratio of 1:5. The suppression potential decreased to around 30% in both media at mBM-MSCs to PBMC ratio of 1:25. Passage 40 cells exhibited even lesser immunosuppression compared to passage 5 cells, where at mBM-MSCs to PBMC ratio of 1:5 mBM-MSCs cultured in DMSS and DFBS suppressed only 40% and 51% of PMBC, respectively. At co-culture ratio of 1:25 the suppression potential of PBMC by mBM-MSCs decreased to around 14% and 6% in DMSS and DFBS, respectively. General comparison between the mBM-MSCs cultured in the two different media showed that the cells grown in DFBS medium had better immunosuppressive activity compared to the cells grown in DMSS medium, although not statistically significant.

Chromosomal analysis

The chromosomal analysis showed significant increase in the chromosome numbers in passage 40 compared to

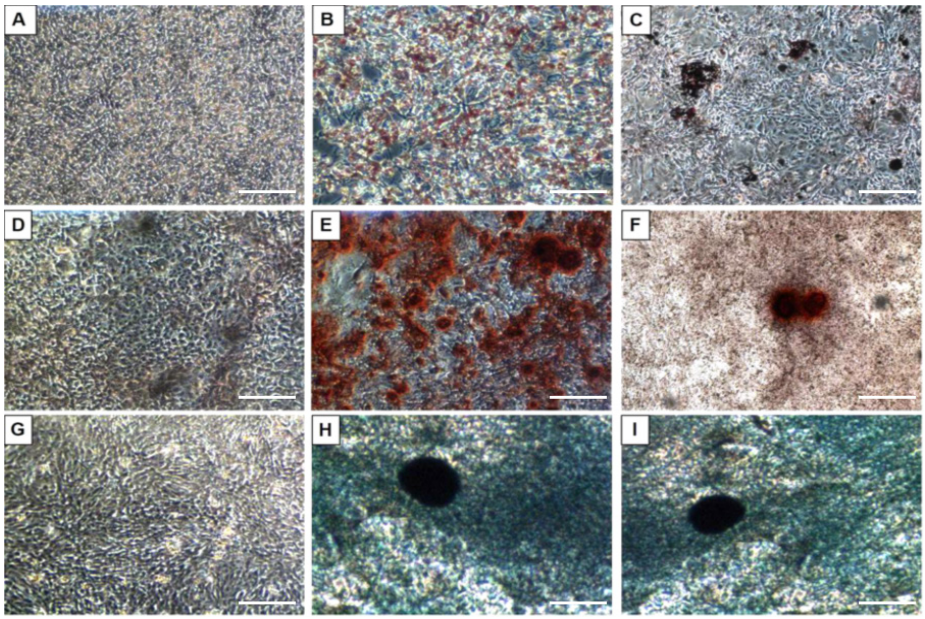

Figure 5: Differentiation assay on mBM-MSCs. Representative images showing the adipogenesis andosteogenesis in the various passages of mBM-MSCs from both DMSS and DFBS media. (A - C) representative images of adipogenesis assay. (A) control, (B) mBM-MSCs cultured in DMSS supplemented medium and (C) DFBS medium after induction with adipogenic media and stained with Oil Red O stain after 21 days. Lipid droplets (stained red) were present in both cultures showing the ability to differentiate into adipocytes. (D - F) representative images of osteogenesis assay. (D) control, (E) mBM-MSCs cultured in DMSS medium and (F) DFBS medium after induction with osteogenic medium and stained with Alizarin Red stain after 21 days. Calcium deposits stained red were present in both cultures showing the ability to differentiate into osteoblasts. (G - I) representative images of chondrogenesis assay. (G) control, (H) mBM-MSCs cultured in DMSS medium and (I) DFBS medium after induction with chondrogenic medium and stained with Alcian Blue stain after 21 days. The proteoglycan in the cartilagous spheroids stained blue, showing the ability of the MSCs in both cultures to differentiate into chondrocytes. Scale bar indicates 100 µm.

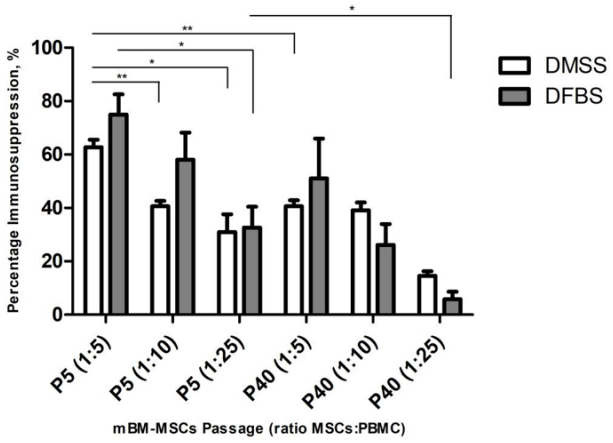

Figure 6: : Immunosuppression assay of mBM-MSCs of different passages and ratios of MSCs to PBMC in both DMSS and DFBS media. Cells were plated at different ratios of mBM-MSCs: PBMC and cultured for 72 hours prior to flow cytometry analysis. Percentage of immunosuppression were obtained by comparing the percentage of proliferation of phytohemagglutinin stimulated PBMC against those co-cultured with mBM-MSCs. Percentage of immunosuppression shown represent mean ± SEM from 3 independent experiments. Statistical analysis using two-way ANOVA and Bonferroni post-hoc test showed that the percentage of immunosuppression was statistically significant when compared between the ratios and passages as indicated in the graph (*p-value ≤ 0.05, **p-value ≤ 0.01). No significant difference was found when compared between the DMSS and DFBS media.

passage 5 in both media (Figure 7). Normal mice have 40 chromosomes, which were observed in passage 5 mBM-MSCs. However, the chromosome number increased to nearly 60 in mBM-MSCs cultured in DMSS medium and slightly above 50 in the cells cultured in DFBS medium.

DISCUSSION

This study points out the interest of using different supplements, in this case, MSS and FBS in culture media for the isolation and expansion of mBM-MSCs and their effects of the MSCs properties. In order to facilitate direct comparison, we have used the same basal media for both supplements. However, one elimination of the study is this approach might not be the best combination for MSS since manufacturers often optimized their supplements for use with their own basal media. But it is still noteworthy to investigate the effects of these supplements on the MSCs culture. So far, there is no specific guideline on the ideal method and complete media formulation for the isolation and growth of MSCs. One of the commonly used supplements for almost all type of cell culture protocol is the fetal bovine serum and in stem cell culture, this serum-based supplement is commonly used at 10-15% (v/v) of the final volume of the complete medium (12). However, there are many issues pertaining to the safety and efficacy of FBS. Besides, FBS exhibited high degree of lot-to-lot variation

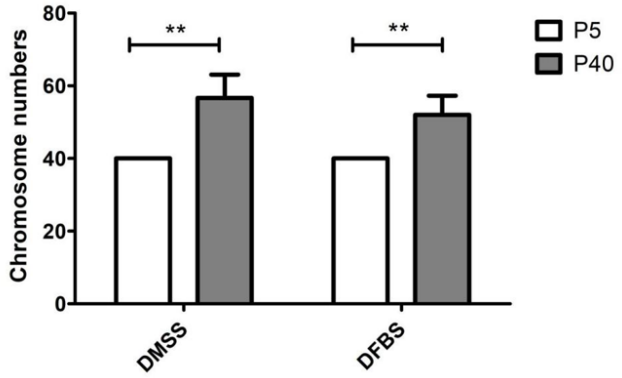

Figure 7: Chromosome analysis on passage 5 and passage 40 mBM-MSCs in DMSS and DFBS media. Chromosome analysis was carried out by counting the chromosome numbers from 20 metaphase spreads. Chromosome numbers represent mean ± SEM from 3 independent experiments. Chromosome numbers increased significantly at passage 40 (P40) compared to the early passage 5 (P5).

causing inconsistencies in the growing condition that will directly influence the biological mechanisms of the cells (20). Recent researches have focused on the application of MSC specialized supplement to replace commonly used FBS as the growth supplement in cell culture media to ensure a more homogenous culture, robust proliferation, colony formation and differentiation (21).

Monitoring the growth and behavior of cells in culture is an important gauge to the performance of the culture medium for specific cells and application. Cell morphology is among the simplest way to evaluate the condition of the cultures merely by observation under the microscope. Proliferation and cell growth rate are also noteworthy indicators. Expression of suitable surface markers via flow cytometry or immunocytochemistry (ICC) should also be examined periodically. MSCs differentiation assays into the appropriate lineages such as osteoblasts, adipocytes and chondrocytes should be performed to confirm the differentiation potential of the cells. For safety assessment, chromosomal analysis should be done routinely to demonstrate that a normal karyotype is retained during the *ex-vivo* expansion (22).

In this study, there were no morphological difference between the MSCs cultured in both media at the early passages as both displayed spindle shape fibroblastic morphology. However, the late passage cells cultured in DFBS acquired epithelial-like morphology, suggesting that the cells might have undergone mesenchymal to epithelial transition (MET). During MET, mesenchymal cells lose their ability to migrate and at the same time acquire cell polarity and adhesion to epithelial layer (23). MET and their reverse process, epithelial mesenchymal transition (EMT), both occur in normal tissues such as gastrulating and regenerating tissues, as well as in transformed tissues and cancers (24). It was proposed that a restrictive mechanism repress this cellular transition in normal adult organisms (25). However, during tumorigenesis, these mechanisms appear to fail, enabling the EMT as described in metastatic cancers. In another study, it was also shown that spontaneously transformed MSCs that was characterized using immunohistopathology, microarray and protein analysis strongly suggest of the dedifferentiation process via MET that led to the transformation process (26). This was also observed in a similar study on long term human skin culture, whereby MET was observed *in vitro* (27).

Next, we assessed the clonogenic and proliferative capacities of MSCs in both media. Clonogenic potential is considered as one of the characteristics of MSCs. Clonogenic efficiency was evaluated by seeding the bone marrow cells at seeding density of $1x10^6$ cells/well in both complete media in triplicates. The number of colonies formed in the DMSS medium was significantly higher and larger than that of DFBS, indicating the clonogenic potential of the cells was significantly increased when cultured using DMSS. The proliferative capacities of MSCs were assessed by their PDT. Cells cultured in DMSS medium showed higher proliferative capacities at passage 0. This indicates that the DMSS media allow faster adaptation and growth especially at the beginning of the isolation stage. DMSS medium was previously shown to be effective in isolating and propagating mBM-MSCs from various mouse strains such as CBA/Ca, ICR and Balb/c (28). Furthermore, MSS is standardized for the culture of mouse mesenchymal stem and progenitor cells which was optimized using cells from C57BL/6 mouse. Standardized and defined components in the culture medium enable the reproducibility and reduces the variability between culture batches. This defined and specialized medium for MSCs growth and expansion was proven in this study to facilitate the proliferation and clonogenicity of MSCs.

Immunophenotyping is another key characterization for MSCs and in this study three MSCs positive markers were used, CD44, Sca-1 and CD106; countered by two negative markers, CD11b and CD45. Due to the heterogeneity of cells isolated from bone marrow, there is no single unique marker that is able to isolate and profile the population of MSCs. The proposed MSCs markers are generally divided into two categories, sole marker and stemness markers. Sole marker is the ultimate MSCs selection tool, by which on its own is sufficient to identify or isolate MSCs from their environment. A stemness marker on the other hand is able to identify a group of MSCs that are highly proliferative and display trilineage potential. Based on the nature of these different markers, sole markers are normally highly expressed, while stemness markers are expressed at a moderate to high level (29). However, it is widely accepted that bone marrow MSCs express CD29, CD44, CD73, CD90, Sca-1 and CD106 (30).

In the current study, expression of CD44 (homing cell

adhesion molecule, HCAM) was consistently high across passages (passage 5 to 40) in both media. CD44, a receptor for hyaluronic acid, mediates the cell-cell and cell-matrix interaction through its affinity to hyaluronic acid. CD44 was implicated in cell-cell interactions, adhesion and migration of MSCs to the bone marrow. Besides that, they are also involved in key cellular processes such as growth, differentiation, motility and survival (31). Therefore, the expression of CD44 remained high throughout the passages as they play an important role in the various cellular processes. This was similarly observed in many other previous studies that showed high level expression of CD44 in *in vitro* culture of MSCs (16).

The expression of another MSCs marker, Sca-1 was also found high throughout the passages (passage 5 to 40) especially in DFBS medium. This was also found in previous studies showing that Sca-1 was highly expressed in isolated MSCs from different mice strains (28,32). Sca-1 like many other MSCs markers, do play crucial roles in cell signaling by regulating key signaling molecules such as Src family kinases (33,34). Besides, it is also widely believed that Sca-1 is involved in receptor-ligand interactions, mediating cell adhesion and signaling. The expression of Sca-1 remains high throughout the passages indicating them BM-MSCs still maintain their stemness till late passages.

Vascular cell adhesion molecules-1 or CD106 is another known adhesion molecule that is expressed by MSCs (35). It was shown that the expression of CD106 is dependent on the cell density of the MSCs, where by the expression was upregulated in MSCs grown in high density (36). In this study, it was observed that the expression of CD106 fluctuated and generally decreases across passages, with a higher expression shown in DMSS medium. This result is similar with previous studies that had also showed that the expression of CD106 is strongly reduced in later passages of MSCs cultured *in vitro* (37,38). Expression of CD106 is also induced by inflammatory cytokines such as tumor necrosis factor (TNF)- α and IL-1β, in particularly during the homing process towards wound or injury site (39). It was speculated that the expression of CD106 decreased in these cultures as there were no external stimuli such as the inflammatory cytokines responsible for the up-regulation of CD106 expression in a well-maintained *in vitro* culture. Furthermore, the *in vitro* microenvironment is totally different with their origin in a body system, whereby interaction between amyriad of chemokines and signaling molecules are lacking in an *in vitro* system.

One of the unique properties of MSCs is their immunomodulatory properties. MSCs were shown to change the function of T cells and B cells, thus exhibiting strong immunosuppressive activity (40). MSCs exhibit their immunomodulatory action via direct cell-cell contact or the secretion of chemical factors or chemokines (41). In order to investigate the immunomodulatory properties of MSCs, immunosuppression assay was carried out on passage 5 and passage 40 mBM-MSCs at different ratios of MSCs to PBMCs. It was observed that early passage mBM-MSCs showed more potent immunosuppresive activity compared to the late passage, indicating some functional changes might have occurred during the prolonged passage in vitro. Decrease in immunosuppresive activity correlated with the decrease of CD106 expression in the late passage of mBM-MSCs, thus giving an indication of the potential regulation of immunosuppressive activities by CD106 as previously reported by Yang and colleagues (2013) (42). In their study, Yang and colleagues managed to show that $CD106^+$ MSCs expressed higher immunomodulating factors such as COX-2, IL-1α, IL-1β, IL-6 and IL-8. Moreover, they also found that the expression of CD106 decreases when passaging *in vitro* (42). In our study, there were no significant difference when the immunosuppressive activities of mBM-MSCs in both complete media were compared. This indicated that mBM-MSCs cultured in both DMSS and DFBS media still had the same immunosuppressive activities at early passage and the potential decreased at late passage. Interestingly, the chromosome analysis revealed unanticipated results. Both the late passage mBM-MSCs in DMSS and DFBS media showed an increase in chromosome numbers which is significantly different than the normal 40 acrocentric chromosomes in mice. This was similar to previous studies showing chromosomal aberrations and the increase in chromosome numbers in transformed murine MSCs (43,44). In this study, chromosomal analysis was only being carried out at passage 5 and 40. In order to have a better insight of when these aberrations start to occur; more in-depth studies have to be carried out. Although the mBM-MSCs cultured in DMSS and DFBS did not show any significant difference in their chromosomal numbers, mBM-MSCs cultured in DMSS were observed to have slightly higher number of chromosomal aberrations. The possible reason behind this is the proliferative capability of mBM-MSCs cultured in DMSS is generally higher than that in DFBS, which in turn lead to increased number of chromosomes. Many other evidences that showed potentially harmful spontaneous transformations of MSCs into sarcomas when cultured *in vitro* had been reviewed by Lye et al. (2016). In comparison, human MSCs was less susceptible to spontaneous transformation as reported before (45). This difference in spontaneous transformation between human MSCs and other animal MSCs can be explained by telomeres. Human telomeric DNA is usually 5–10 kb long, while mouse and rat have longer telomeric length of 30–100 kb (46). The longer telomeres in mouse allow cells to proliferate for many generations before reaching their Hayflick limit, thus, increasing the risk of genetic aberrations (47). Therefore, this study provided support to the claims that mBM-MSCs do exhibit chromosomal aberration under prolonged culture in vitro.

CONCLUSION

In the current study, we have shown that mBM-MSCs can be successfully isolated and expanded in both DMSS and DFBS media. In general, DMSS medium was shown to be better in derivation and expansion of mBM-MSCs. As expected DMSS gave higher number of CFU, lower PDT value and greater differentiation efficacy. Regardless of those differences, mBM-MSCs in both media showed similar CPDL, immunophenotype and immunosuppressive activity profiles. Although the stemness characteristics and immunosuppression potential of mBM-MSCs preserved in both media, abnormal number of chromosomes was found in the prolonged cultures. Further investigations at the molecular level and *in vivo* study should be conducted to gain better insights of these transformed mBM-MSCs. Limitation of this study is the usage of passage 40 cells to represent the late passage. Systematically investigating every alternate passages would reveal more information on the progression of the spontaneous transformation occuring during the prolong passaging of these cells, thus giving an idea on which passages are genetically safe to work with for therapy. This study also gives an emphasis that early passage mBM-MSCs are more reliable to be used for downstream experiments for preclinical regenerative medicine and therapy study.

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