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Local manufacturing processes contribute to variability in human mesenchymal stromal cell expansion while growth media supplements contribute to variability in gene expression and cell function: a Biomedical Excellence for Safer Transfusion (BEST) collaborative study

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ABSTRACT

Background aims: Culture-derived mesenchymal stromal cells (MSCs) exhibit variable characteristics when manufactured using different methods, source material and culture media. The purpose of this multicenter study was to assess the impact on MSC expansion, gene expression and other characteristics when different laboratories expanded MSCs from cultures initiated with bone marrow−MSC aliquots derived from the same donor source material yet with different growth media.

Methods: Eight centers expanded MSCs using four human platelet lysate (HPL) and one fetal bovine serum (FBS) products as media supplements. The expanded cells were taken through two passages then assessed for cell count, viability, doubling time, immunophenotype, cell function, immunosuppression and gene expression. Results were analyzed by growth media and by center.

Results: Center methodologies varied by their local seeding density, feeding regimen, inoculation density, base media and other growth media features (antibiotics, glutamine, serum). Doubling times were more dependent on center than on media supplements. Two centers had appropriate immunophenotyping showing all MSC cultures were positive for CD105, CD73, CD90 and negative for CD34, CD45, CD14, HLA-DR. MSCs cultured in media supplemented with FBS compared with HPL featured greater T-cell inhibition potential. Gene expression analysis showed greater impact of the type of media supplement (HPL versus FBS) than the manufacturing center. Specifically, nine genes were decreased in expression and six increased when combining the four HPL-grown MSCs versus FBS (false discovery rate [FDR] <0.01), however, without significant difference between different sources of HPL (FDR <0.01).

Conclusions: Local manufacturing process plays a critical role in MSC expansion while growth media may influence function and gene expression. All HPL and FBS products supported cell growth.

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Introduction

Due to the regenerative medicine and immune-modulatory properties of mesenchymal stromal cells (MSCs), they are a promising future treatment for a large number of diseases. MSCs are usually obtained from umbilical cord tissue, bone marrow or adipose tissue, but they must be expanded in culture to obtain enough cells for clinical applications [1,2]. Classically, MSCs have been cultured in media supplemented with fetal bovine serum (FBS). However, FBS could result in the transmission of variant Creutzfeldt−Jakob disease or other xenogeneic infections, and together with animal welfare reasons, it is preferred that FBS or other animal-derived products not be used for the production of clinical cell therapies intended for human use [3]. Therefore, this study assessed the use of human platelet lysate (HPL) as an alternative supplement for MSC production.

HPL is prepared from platelet products that had been collected and manufactured for blood transfusion but have expired. Many of these HPL products are commercially available, and some are available for use in clinical trials [4,5]. HPL-manufacturing protocols use different starting platelet products (i.e., buffy coat or apheresis platelet products, with or without pathogen inactivation) and are prepared using different manufacturing methods. Currently, there is no consensus on which HPL preparations are best for MSC production. In addition, HPL reportedly offers equivalent or better growth support for MSC cultures than FBS [6−10]. HPL is also being tested for its capacity to expand other cell types of potential therapeutic value [11]. However, HPL manufacturing is not standardized, and different HPL formulations may have variable impact on MSC cultures.

We reported previously that functional and molecular differences existed among MSC preparations that were produced by different centers using the same bone marrow aspirate as starting material for MSC production [12]. The goal of this study was to evaluate the *in vitro* expansion of MSCs when using different sources of HPL products versus FBS as growth media supplements (GMS). To assess the relevance of the local manufacturing process as well as the impact of using different sources of HPL, we compared culture-derived MSCs that were derived from the same source material that had been cultured with four different HPL products or with FBS by eight different international manufacturing centers. We analyzed growth kinetics and gene expression as well as MSC functions such as immunomodulation. Each center used their standard method for culturing MSCs. Each center measured the expression levels of their culture-derived MSC surface markers, but MSC functional and gene expression analyses were sent to and performed by a central laboratory.

Methods

Study design

Frozen vials aliquoted from a single lot of bone marrow−derived MSCs prepared in one laboratory were distributed from a central laboratory to each participating center. Each participating center cultured the MSCs with four different sources of HPL and a single source of FBS. The cells were harvested after two passages when the cultures reached a 70−80% confluency. Each laboratory assessed MSC growth and expansion and tested the cells for the expression of MSC phenotype markers using their internal assays. In addition, each center sent samples of cryopreserved MSCs to centralized testing laboratory for evaluation of cell function and gene expression (supplementary Figure 1).

MSC starting product

Each participating center received two vials containing 1 million cells of bone marrow−derived MSCs. MSCs were from a single donor (a 22-year-old woman) at passage 1 were obtained from Rooster Bio

Inc. (Frederick, MD, USA) by one of the participating sites, who then expanded the MSCs to passage 2. with The second passage of MSCs were generated using the Quantum Bioreactor Platform (Terumo BCT, Lakewood, CO, USA) with MEM alpha (Lonza, Walkersville, MD, USA) supplemented with 10% FBS (Life Technologies, Carlsbad, CA, USA). Cells were qualified as MSCs using a human MSC analysis kit and flow cytometry (BD Biosciences, San Jose, CA, USA). Second-passage cells were positive for CD105, CD90, CD73 and negative for CD45, CD34, CD19, CD11b and HLA-DR. Cells at the passage 2 generation were then cryopreserved and distributed to the participating centers.

Study centers

Eight Biomedical Excellence for Safer Transfusion (BEST) Collaboration centers were selected to participate: New York Blood Center (NYBC), Vitalant, University of Minnesota, National Institutes of Health (NIH), Japanese Red Cross, University of Maryland, Australia Red Cross Lifeblood and the University of Utah. Each center was provided two vials of second-passaged MSCs, aliquots of each of four sources of HPL and a single source of FBS, a study protocol and expansion protocol.

Growth media supplements

HPL products

Four different sources of HPL (Compass PLUS, Mill Creek PLTMax, Australia Red Cross Lifeblood buffy coat platelet derived HPL, NYBC Plasate) and one source of FBS (HyClone) were used in this study. All four sources of HPL and the single source of FBS were received by one center, aliquoted and distributed to each of the eight participating centers. The HPL and FBS included the following:

Compass PLUS (Compass Biomedical, Hopkinton, MA, USA): Heparin-free, research-grade, commercially manufactured HPL, made from large pools of expired transfusion-grade apheresis platelets from Food and Drug Administration (FDA)-registered/Association for the Advancement of Blood & Biotherapies (AABB)-accredited blood centers (https://www.compassbiomed.com/plus.html).

Mill Creek PLTMax (Mill Creek Life Sciences, LLC, Rochester, MN, USA): Research-grade, commercially manufactured, pooled HPL, made from large pools of expired transfusion-grade apheresis platelets from FDA-registered/AABB-accredited blood centers (https://www.millcreekls.com/pltmax).

Australia Red Cross Lifeblood buffy coat platelet−derived HPL (South Melbourne, Victoria, Australia): Australian Red Cross Lifeblood−produced research-grade HPL product from pools of 10 clinical-grade, expired buffy coat−derived platelets, which were resuspended in 30% plasma and 70% platelet additive solution. The platelets were prepared under standard current Good Manufacturing Practice manufacturing conditions.

NYBC Plasate (New York Blood Center, New York, NY, USA): A research-grade product from small pools of expired transfusion-grade apheresis platelets in 100% plasma that were collected by FDA-registered/AABB-accredited blood centers. (https://www.nybc.org/products-and-services/cellular-therapy/cellular-therapy-products/plasate-human-platelet-lysate-research-and-gmp-grade/).

FBS product

HyClone (Cytiva, Marlborough, MA, USA) standard FBS of US origin (https://www.cytivalifesciences.com/en/us/about-us/our-brands/hyclone).

Expansion protocol

Centers were instructed to thaw, count and pool both vials of MSCs, and then seed the MSCs into 96-well plates to run all

experiments in duplicate for each condition. All HPL sources were to be used at a concentration of 10%, and FBS was to be used according to each institution's in-house expansion protocol. Centers expanded the source MSCs (i.e., second-passage MSCs) through two additional passages as follows: The MSCs were seeded, cultured to 70−80% confluency, harvested, counted and tested for viability; the third-passage MSCs were then used to seed the next passage, which were grown again to 70−80% confluency. At the end of the culture period, cells were harvested. A portion of the cells were used in-house to perform cell counts and viabilities and the remainder were used for additional testing or further expanded for testing (immunophenotyping, gene expression and immunosuppression).

Assays

Each institution performed cell count, viability, proliferation assays and immunophenotyping (CD105, CD73, CD90, CD34, CD45, CD14, HLA-DR) [13] according to each center's preferred methodology (supplementary Table 1). The number of population doubling times (PDTs) were calculated after each passage. PDT was calculated using the formula to follow:

$$\text{Doubling time} = (\times \text{ number of hours since plated})$$
$$\times (\text{Log}(2))/(\text{Log}(\text{Fold Expansion}))$$

The remaining cells were expanded until 15 million MSCs were achieved. These cells were cryopreserved in CS10 (BioLife Solutions, Bothell, WA, USA) according to the manufacturer`s instructions (https://www.biolifesolutions.com/wp-content/uploads/2018/01/6012_07-CryoStor-Product-Information-Sheet.pdf) at 10 million and 5 million cells per vial. The frozen aliquots were stored at $-160°C$ (liquid nitrogen), except in Japan MSCs were stored at $-80°C$, and were shipped on dry ice to a central laboratory for T-cell suppression and gene expression studies.

Proliferation assay

A protocol for the MTT assay was provided to all centers. MSCs were grown separately with the four sources of HPL and the single source of FBS, in a 96-well plate, and assayed for replication by Thermo Fisher Scientific's Vybrant MTT Cell Proliferation Assay. The compound MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) is solubilized in PBS and then introduced to serum supplemented growth media, cells are then cultured in the MTT-Serum media for 4 h to allow the cells to incorporate the compound and convert it to insoluble formazan. After the culture period, sodium dodecyl sulfate is added to each well to disrupt the cell membrane, release the formazan and assay the wells for absorbance at 570 nm. Greater absorbance values indicate increased number of cells and greater proliferation.

T-cell suppression assay

The immunosuppressive properties of MSCs were evaluated using a mixed lymphocyte reaction assay (SAIC-Frederick, Frederick, MD, USA) that was performed by a central laboratory. Ficoll-separated peripheral blood mononuclear cells (PBMCs) were plated in 96-well plates at 1×10^5 responder cells per well. Responder cells were co-cultured with 2500 cGy of irradiated stimulator PBMCs at a concentration of 1×10^5 cells per well. MSCs from the different centers were added at concentrations of 1×10^4, 4×10^4 and 10×10^4 cells/well, only the concentration of 4×10^4 cells/well is reported. Culture plates were incubated for 6 days in a humidified 5% CO_2 incubator at 37°C. On the day of harvest, 0.5 μCi of 3H-thymidine (3H-TdR) was added to each well for 4 h with lymphocyte proliferation measured using a liquid scintillation counter. The effect of MSCs on the mixed lymphocyte reaction was calculated as the percentage of the suppression that occurred as with MSCs compared with the proliferative response of the control without MSCs. The control was set to 0% suppression. The experiments were performed in replicates of three for each variable tested. Seventy percent suppression was deemed acceptable and consistent with MSCs used in clinical trials [14].

Gene expression analysis

Gene expression microarray was performed at a central laboratory (NIH). Total RNA extractions were performed on the MSC samples using RNeasy Mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer's protocol. RNA was quantified using Nanodrop 8000 (Thermo Fisher Scientific, Wilmington, DE, USA). Total RNA integrity was evaluated following isolation using a 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). Samples with an RNA Integrity Number value ≥ 8 were used for gene expression analysis.

Gene expression profiling was performed on $4 \times 44K$ Whole Human Genome Microarrays (Agilent , Santa Clara, CA, USA) according to the manufacturer's instructions. In general, 200 ng of total RNA from each sample was amplified, labeled and hybridized on the array chip using a Quick Amp Labeling kit (Agilent). Array images were obtained using an Agilent Scanner G2600D and were extracted using Feature Extraction 12.0 software (Agilent). Partek Genomic Suite 6.4 (Partek Inc., St. Louis, MO, USA) was used for data visualization.

Statistical and microarray data analysis

Principal component analysis (PCA) was performed using the R software (version 4.0.2, www.R-project.org) built-in function "prcomp" [15], to visualize the similarities and differences among the samples from different centers. Unsupervised hierarchical clustering was performed by the R software (version 4.0.2) built-in function " hclust" using all the genes on the array to group similar samples into clusters Differentially expressed genes between two groups (HPL vs FBS) were determined by Student's t-test and adjusted by the Benjamini−Hochberg method using R software (version 4.0.2) and were presented in heatmap using Cluster and TreeView Software [16].

Pathway analysis

The Gene Ontology (GO) knowledgebase (including biological process, molecular function and cellular component) enrichment analyses were subsequently conducted on the differentially expressed genes using the DAVID software [17]. Donut distribution charts of the significant GO pathways ($P < 0.05$) were generated using the "Plotly" package in the R software. Pathways are presented in with the name, P-values, then the number of genes found, followed by the percentage of those genes in the GO terms.

Results

A summary of the results obtained for cell counts, viabilities, doubling times and immunosuppression is presented in Table 1. In comparing GMS, cell counts on average were greater with shorter doubling times for all HPL as compared with FBS-supplemented MSC cultures. MSC viabilities on average were similar and independent of the GMS. On average, using FBS resulted in greater immunosuppression. When comparing centers, there was variability in cell count, viability, PDTs, immunosuppression capacities as well as in immunophenotyping.

The local manufacturing process is more relevant for MSC dose production efficiency than GMS

To identify the main denominators for MSC production efficiency, we compared the growth kinetics (defined as PDTs) of bone

Table 1
MSC culture results by GMS and center.

	Cell count, $\times10^6$	Viability, %	Doubling time, h	Immunosuppression,%	Immunosuppression pass $\geq$70%[a]
GMS					
FBS	4.28 (1.99−9.37)	95 (91−99)	87 (28−285)	72 (39−90)	3/5
HPL C	8.05 (1.29−16.35)	95 (85−99)	71 (28−204)	36 (14−76)	1/5
HPL MC	7.23 (3.11−16.55)	94 (88−98)	66 (25−199)	48 (23−68)	1/6
HPL ARC	6.97 (2.28−15.30)	95 (89−99)	82 (19−241)	47 (32−74)	1/6
HPL NYBC	8.75 (1.83−23.15)	94 (85−99)	50 (30−109)	36 (8−81)	1/6
Center					
NYBC	4.97 (3.52−5.89)	92 (89−95)	36 (33−42)	38 (8−86)	1/5
Maryland	11.08 (8.72−13.76)	95 (91−99)	39 (34−54)	ND	ND
Australia	3.50 (2.09−5.62)	98 (97−99)	38 (31−48)	38 (16−80)	1/5
Japan	16.14 (9.37−23.15)	94 (93−96)	207 (109−285)	65 (27−90)	1/5
NIH	2.61 (2.07−12.90)	88 (85−91)	26 (19−30)	26 (16−39)	0/5
Minnesota	8.06 (2.21−4.59)	96 (93−98)	34 (28−43)	69 (34−90)	4/5
Vitalant	2.90 (2.21−4.59)	96 (95−97)	119 (75−144)	55 (32−78)^	0/3

ARC, Australia Red Cross; C, Compass; FBS, fetal bovine serum; GMS, growth media supplements; HPL, human platelet lysate; MC, Mill Creek; MSC, mesenchymal stromal cells; ND, not done; NIH, National Institutes of Health; NYBC, New York Blood Center. ^no FBS
[a] T-cell inhibition ≥70% passing.

marrow−MSCs from the same donor cultured with different media supplements at different center. To assess the general relevance of the local manufacturing process after source second-passaged MSCs underwent two additional passages, the centers applied their individual MSC seeding protocols and densities. For passage 3, the centers used the same seeding density for all GMS, with variations between centers. For passage 4, some centers kept the seeding density consistent for all GMS while others varied the densities. The variable "center" remained the main discriminator for MSC expansion potential. In both consistent and variable densities for MSC seeding, the overall PDT patterns remained similar for each center, but varied between the centers. We observed a minor variability between FBS and HPLs as well as between the HPL sources, but substantial differences between the PDTs of the individual centers (Figure 1A,B). These data suggest the local manufacturing process as the most relevant factor for MSC expansion with a higher impact than GMSs, such as HPL or FBS.

GMS impact MSCs' gene expression more than the manufacturing location

Global gene expression was analyzed using microarrays in 33 MSC samples prepared by seven centers at a central laboratory. The University of Utah laboratory provided four MSCs samples (three cultured with HPL and one cultured with FBS). The Vitalant laboratory provided four samples (all cultured in HPL). The other five laboratories provided five samples (four cultured in HPL and one cultured in FBS). The PCA was performed on 29 MSC samples from six manufacturing centers with each providing four to five MSC samples. The Vitalant center was excluded from the analysis, as the FBS sample was not provided. The PCA for the entire gene expression set showed that, except for one center, the MSCs cultured with FBS were set distinctly apart from the MSCs cultured with HPL, notably regardless of HPL source and manufacturing center (Figure 2A). The MSCs cultured with FBS at the center in Japan grouped within all other samples grown with HPL, suggesting a similar gene expression pattern, which was confirmed by the hierarchical clustering analysis (Figure 2B).

The detailed pairwise comparison of the HPL samples from the seven manufacturing centers identified only a relatively small number of genes being differentially expressed, suggesting no signfiicant difference in gene expresson at diffferent manufacturing centers (supplementary Table 2). There were also no significant difference in gene expression when comparing different sources of HPL: in pairwise comparison of four HPL sources, 0 genes were differentially expressed based on false discovery rate (FDR) <0.01. The comparison
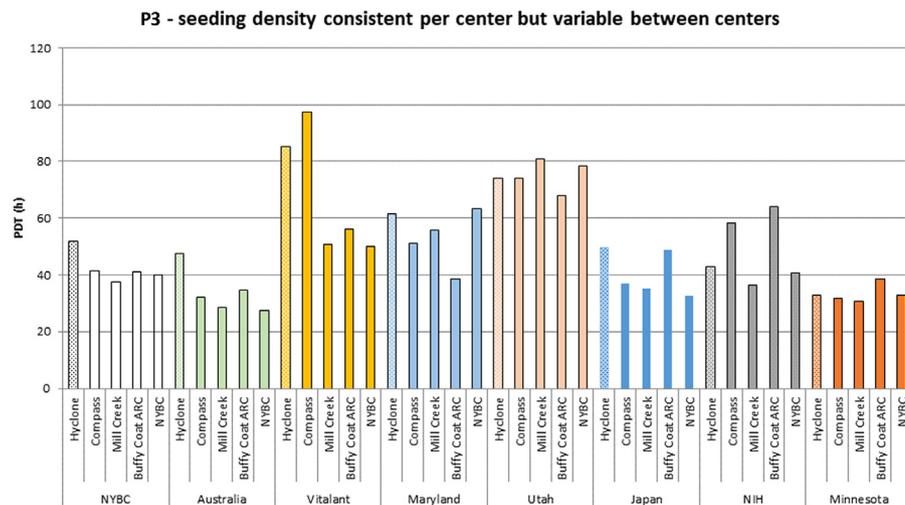

Figure 1. Growth kinetics of MSCs. PDTs of MSCs from the same donor cultured with different media supplements at different centers. (A) Assessment of the general relevance of the local manufacturing process at P3: seeding density consistent per manufacturing center, but variable between centers. (B) Investigation of the relevance of the respective MSC seeding densities on the observed growth differences at P4: seeding densities variable between manufacturing centers. Green centers: seeding density consistent; red centers: seeding density inconsistent. (Color version of figure is available online.)
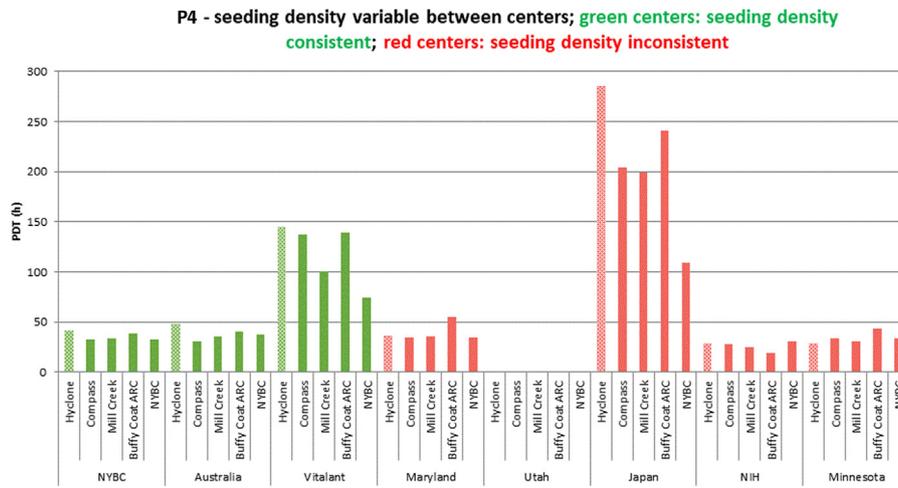
Figure 1. Continued.

of pooled HPL data (four HPLs combined) versus FBS revealed 30 genes being down- and 34 genes upregulated (FDR <0.05), or nine down- and six upregulated genes (FDR <0.01, Figure 3A, supplementary Table 3). DAVID pathway analysis related to these genes identified processes linked to ligase activity, ubiquitin-dependent activities, protein catabolism, peripheral nervous system development and oligodendrocyte differentiation (down regulated by HPL) as well as interferon production and replicative senescence (upregulated by HPL) (Figure 3B,C).

Of note, we observed very few differences in gene expression between MSC samples according to the type of HPL media supplement.

In analyzing gene regulation differences being shared by each comparison HPL versus FBS, there were 25 down- and 13 upregulated genes (Figure 4A, supplementary Table 4). Pathways related to these genes being downregulated by HPL were Notch signaling, extracellular regions and identical protein binding, whereas phagocytotic vesicle memebrane, lipopolysacchride signaling, interferon-beta and interleukin-8 production-related processes were upregulated by HPL (Figure 4B,C).

Taken together, the transcriptome analysis revealed the discriminator hierarchy FBS greater than manufacturing centers with only small/inconsistent differences between the HPL groups.

The immunosuppressive potential of MSCs is affected by both media supplements and manufacturing process

Next, we aimed to investigate the potential effects of media supplements and manufacturing process on MSC function, defined as T-cell immunosuppressive potential. We tested the capacity of MSCs to suppress PBMC proliferation, which is currently regarded as a surrogate test to assess the MSCs′ immunomodulation potential. The MSCs from all centers cultured with FBS and HPL showed a robust potential to suppress PBMC proliferation *in vitro* with a trend to stronger suppression when the MSCs were cultured with FBS (average 65%, range
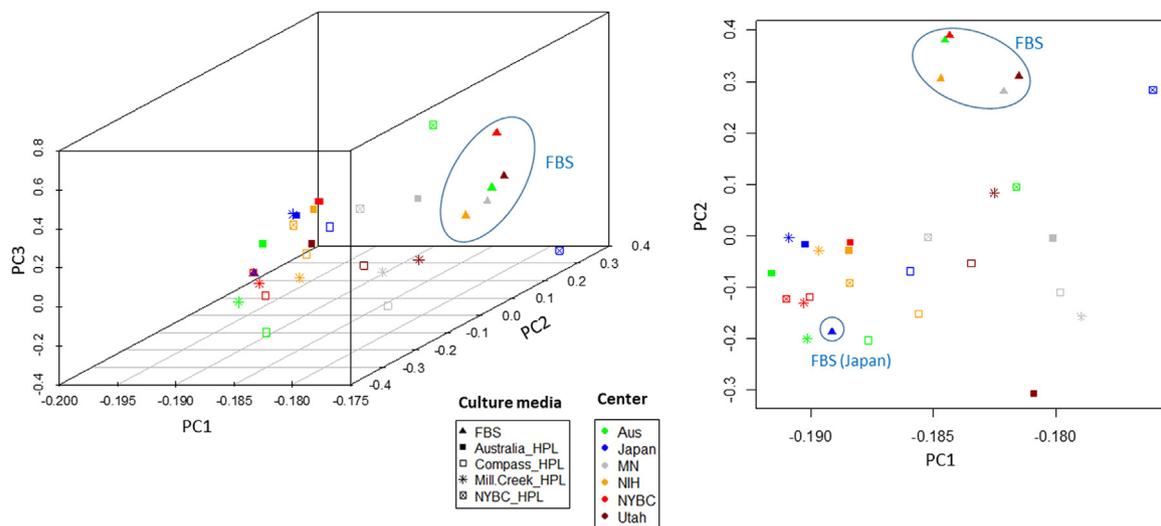

Figure 2. MSCs unsupervised gene expression analyses. (A) PCA of gene expressed by MSCs produced at different centers using growth media supplemented with FBS or HPL. Data from the 29 MSCs samples produced by the six centers were analyzed (Vitalant center was excluded due to lack of FBS sample). Left: three-dimensional PCA using PC1, PC2 and PC3. Right: two-dimensional PCA using PC1 and PC2. Each center is represented by a different colored circle. Each HPL is represented by a different shape. (B) Hierarchical clustering analysis of genes expressed by MSCs. Data from the 29 MSCs samples produced by the six centers were analyzed (Vitalant center was excluded due to lack of FBS sample) by unsupervised hierarchical clustering analysis using all the genes on the microarray. Similar clustering was observed when using the top 1000 variance genes across all samples. The manufacturing center and type of media supplement, HPL type or FBS, as indicated. (Color version of figure is available online.)
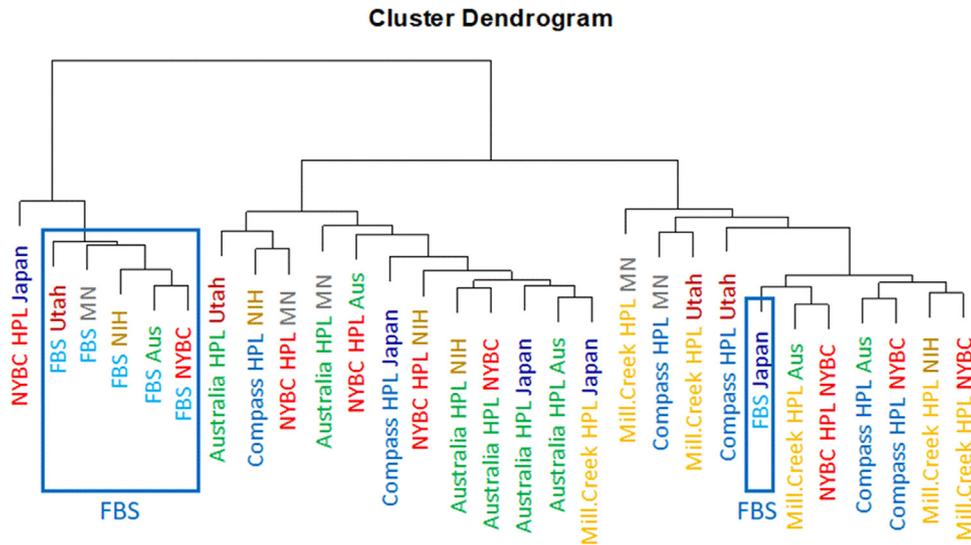
Cluster Dendrogram

Figure 2. Continued.

8−81%) compared with HPL (average 43%, range 27−90%) (Figure 5, Table 1). In addition, the suppressive potential of the MSCs varied between the different manufacturing centers, with one center obtaining a greater rate of passing (defined as >70%) results.

MSC phenotyping

Likely due to technical issues with flow cytometry analyses at some centers and inconsistent reporting, it was challenging to compare the MSC identity marker expressions between all centers and media supplements (data not shown). Two centers did not report flow cytometry results (NIH and Minnesota). Consistent flow cytometry data sets from two centers (NYBC, Australia) showed positivity for CD73, CD90 and CD105 (>70% positive) and negativity for CD14, CD34, CD45 and HLA-DR (<10% positive) confirming MSC identity criteria. If applying the International Society for Cell & Gene Therapy criteria (>90% positive for CD73, CD90, CD105 and <2% positive for CD45, CD34, CD14, HLA-DR) [13], one site had all MSC cultures pass (Australia), whereas the other FBS and HPL Compass passed (NYBC). One site reported MSCs with all GMS to be CD34 positive (Vitalant); one center (Maryland) had low expression of CD105 and CD90; one center (Utah) had invalid results for CD105 and was not able to perform testing on one HPL (NYBC) MSC culture; one center (Japan) had low CD90 expression with two HPLs (Mill
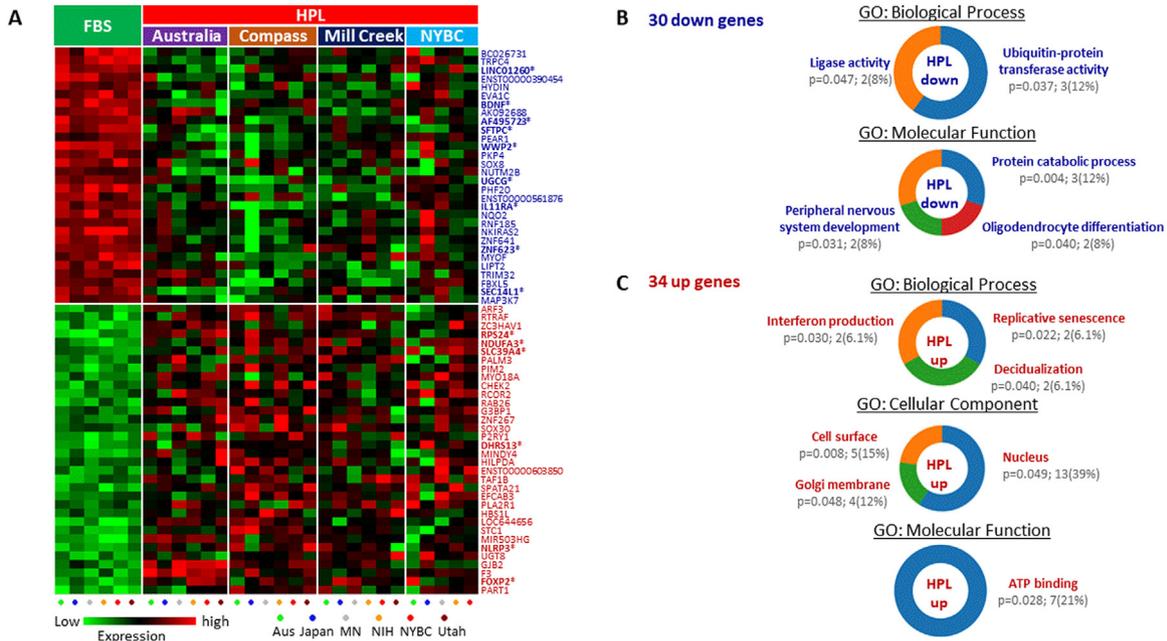

Figure 3. Supervised analysis for pooled HPL versus FBS. (A) The gene expression profiling from the 23 pooled HPL samples were compared with the six FBS samples using the *t*-test. Thirty genes were identified as downregulated and 34 genes upregulated in the pooled HPL versus FBS based on FDR <0.05. Nine genes were downregulated and six genes upregulated based on FDR <0.01 (those 15 genes were bold highlighted and noted by asterisk in the heatmap). Red color represents relatively high expression, and green color represents relatively low expression. Detailed gene list and associated fold change and *P* value are provided in supplementary Table 3. (B−C) Pathway analysis for those 30 downregulated genes (B) and 34 upregulated genes (C) in HPL versus FBS, using GO datasets. Pathway name, *P*-values, the number of genes found, and the percentage of those genes in the GO terms are shown in the donut chart. (Color version of figure is available online.)
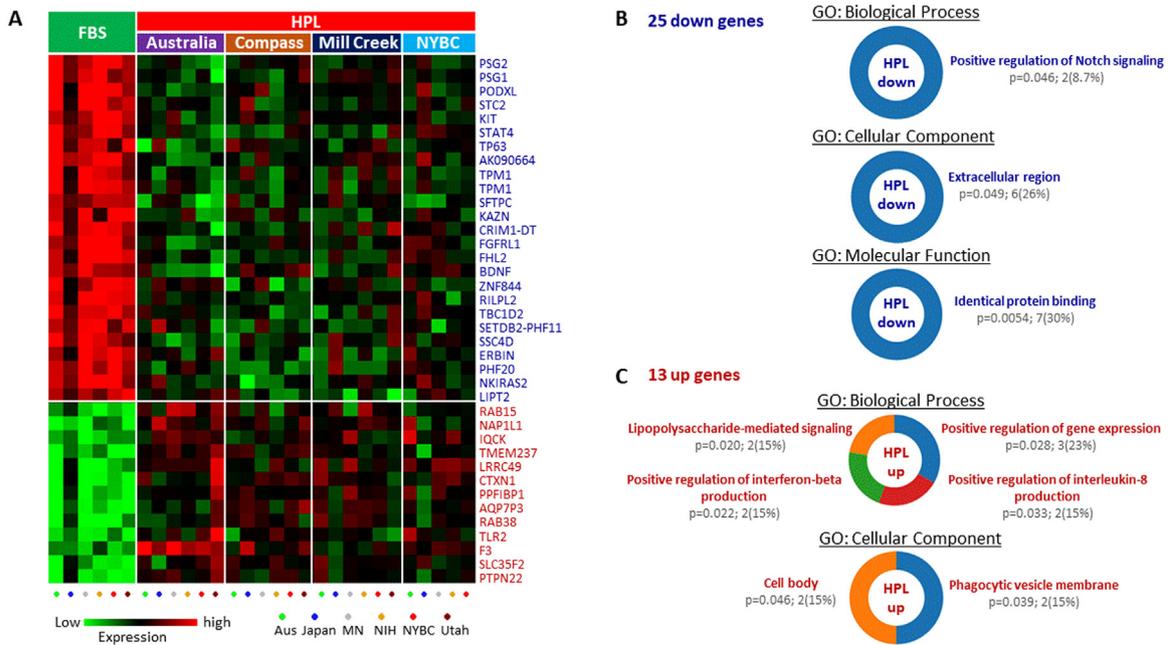
Figure 4. Supervised analysis for individual HPL media versus FBS. (A) The gene expression profiling for each HPL media (Australia, Compass, Mill Creek, NYBC) were compared with the six FBS samples using t-test, to see the different HPL media effect. Each HPL media versus FBS generated a significant gene list ($P < 0.01$). Then, we compared the four significant gene lists from the four HPL media versus FBS, and found 25 genes were constantly downregulated in all 4 HPL and 13 genes were constantly upregulated in all 4 HPL ($P < 0.01$). In heatmap, red color represents relatively high expression and green color represents relatively low expression. Detailed gene list and associated fold change and p value are provided in supplementary Table 4.

(B–C) Pathway analysis for those 25 downregulated genes (B) and 13 upregulated genes (C) shared by each HPL media versus FBS, using GO datasets. Pathway name, *P*-values, the number of genes found and the percentage of those genes in the GO terms are shown in the donut chart. (Color version of figure is available online.)

Creek and ARC). Thus, although four centers performed flow cytometry, there were a number of technical challenges and inconsistent reporting.

MTT assay showed limited performance

Three centers performed the MTT assay. NYBC showed that HPL had 1.8 (1.5−2.1) greater (MTT result of HPL/FBS) metabolic activity than FBS, whereas Utah and NIH indicated greater metabolic activity for FBS (NIH: HPLs were 0.4, range 0.2−0.6, lower to FBS; Utah: HPLs were 0.7 [only 3 reported], range 0.5−0.8, lower to FBS).

Discussion

Both the HPL groups and FBS group demonstrated an ability to support expansion of MSCs at multiple locations worldwide. The local manufacturing process contributed to more variability in expansion, whereas the GMS contributed to more variability in gene expression and cell function. Each manufacturing location used their specific protocols, likely contributing to the expansion variability.

The BEST collaborative previously demonstrated the variability of gene expression and function when MSCs are manufactured at different centers [12]. This follow-up study aimed to first control more variables in the manufacturing process yet compare the impact of
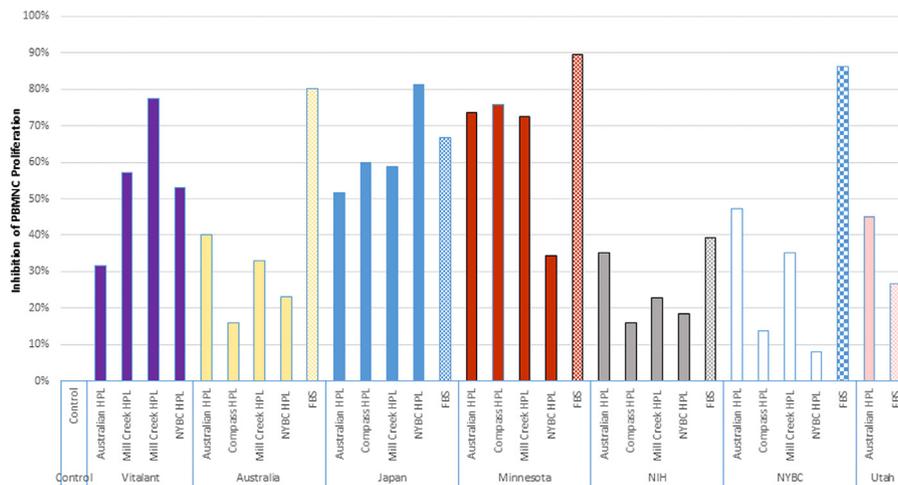

Figure 5. Immunomodulation potential of MSCs. The potential of MSCs to suppress PBMC proliferation was used as a surrogate for their immunomodulation capacity. The graph shows the inhibition of PBMC proliferation in percent after 6 days co-culture of 1×10^5 PBMCs with 4×10^4 MSCs. (Color version of figure is available online.)

different GMS. However, as shown herein, local manufacturing differences continue to impact MSC characteristics. Differences in MSC-manufacturing protocols among laboratories need to be further explored, as they may result in challenges to developing multicenter manufacturing studies as well as differences in impact on patient outcomes during clinical trials [18]. As such, future studies addressing multicenter manufacturing to mitigate variation need to be performed.

Our findings support the use of HPL for MSC manufacturing instead of FBS, which enables the elimination of animal products from human cell manufacturing. This is with the caveat that FBS overall resulted in greater immunosuppressive function and also that HPL altered gene expression differently than FBS, which could impact functionality. This has yet to be explored. If MSCs are administered clinically for the purpose of immunosuppression, this may have clinical impact, specifically demanding to develop adequate dosing strategies to obtain equivalent immunosuppressive potential. Our results also show similar MSC culture outcomes with different sources of HPL products used, including those made from buffy coat versus apheresis platelets, as well as from platelets stored in 100% plasma and 70% platelet additive solution. Differences in doubling times were more dependent on center than GMS. This is likely due to variation in the manufacturing protocols and warrants further detailed investigation.

To decrease the known variablity of MSCs due to manufacturing, the International Society for Cell & Gene Therapy set out recommendations for bone marrow−MSC manufacturing [13]. There has also been progress in defining appropriate assessment of potency as well as purity (immunophenotyping) and other product characteristics [19]. In this study, we used PBMC immunosuppression to assess MSC potency. Overall, FBS resulted in more "potent" MSCs regarding *in vitro* immunosuppression. However, here again a center effect was noted as MSCs manufactured at Minnesota were more likely to stronger suppress PBMC proliferation *in vitro*. Yet, it cannot be ruled out that the need to ship the MSCs to a central center resulted in lower potency recordings. Using a similar assay, it has been demonstrated that both HPL and FBS result in decreased proliferation [20].

Global gene expression differed more between FBS and HPL than between manufacturing centers or by GMS. Notably the number of significant differences were relatively small compared with the number of genes tested. Specifically, only 30 genes were downregulated and 34 upregulated in HPL versus FBS using FDR <0.05. When decreasing FDR to <0.01, only nine genes were downregulated and 6 upregulated in HPL compared to FBS. We found some impact of GMS on various pathways such as Notch signaling, extracellular regions, identical protein binding, phagocytotic vesicle memebrane, lipopolysacchride signaling, interferon-beta and interleukin-8 production-related processes. Moreover, we identified a center-related influence on the *in vitro* proliferation capacity of the MSCs. However, the clinical significance of these findings are unknown and have yet need to be explored. investigated in further studies. In addition, given that MSCs are being used in a wide variety of diseases, the optimal manufacturing process may differ depending upon indication.

There were a number of limitations of this study. First, due to the local challenges with immunophenotyping, it is difficult to assess purity of MSCs in this study. The immunophenotyping results had multiple invalid results and wide variability resulting from technical challenges. Therefore, it is suggested to develop a robust flow cytometry platform, comparable with the ISHAGE protocol for CD34-positive enumeration, for the MSC quality control matrix [21]. Second, seeding densities varied between centers for first expansion and then at some centers varied between GMS at the second seeding. Thus, this makes it difficult to compare these expansions. Next, local manufacturing process was the main variability. Future studies should aim to control more variables in the local manufacturing process. However, the goal of this study was to compare HPL/FBS with

each center using their own protocol, but this resulted in wider variation than expected.

In summary, our findings suggest that HPL may be a suitable supplement to expand MSCs; however, standards for expansion may be necessary to decrease center dependent variability. In addition, we identified that GMS may further contribute to MSC culture variability. To elucidate the impact of MSC variability both from local manufacturing and from GMS, additional studies are warranted. In order to control for local manufacturing variability in the future, more stringent processes need to be defined. The results of our findings suggest that clearly defined manufacturing and release criteria are needed to control for variability, which may have clinical impact.

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Declaration of Competing Interest

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

Author Contributions

Conception and design of the study: BHS, RS, MJF, PJN, DHM, JR, DS, MT, DM, SP. Acquisition of data: BHS, MJF, PJN, DHM, PJ, JR, DS, MT, DM. Analysis and interpretation of data: BHS, RS, MJF, PJN, DHM, PJ, JR, DS, MT, DM, HG, SP. Drafting or revising the manuscript: BHS, RS, MJF, PJN, DHM, JR, DS, MT, HG, SP. All authors have approved the final article.

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Supplementary materials

Supplementary material associated with this article can be found in the online version at doi:10.1016/j.jcyt.2023.11.003.

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