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Scalability and process transfer of mesenchymal stromal cell production from monolayer to microcarrier culture using human platelet lysate

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Abstract

Background aims. The selection of medium and associated reagents for human mesenchymal stromal cell (hMSC) culture forms an integral part of manufacturing process development and must be suitable for multiple process scales and expansion technologies. *Methods.* In this work, we have expanded BM-hMSCs in fetal bovine serum (FBS)- and human platelet lysate (HPL)-containing media in both a monolayer and a suspension-based microcarrier process. *Results.* The introduction of HPL into the monolayer process increased the BM-hMSC growth rate at the first experimental passage by 0.049 day and 0.127/day for the two BM-hMSC donors compared with the FBS-based monolayer process. This increase in growth rate in HPL-containing medium was associated with an increase in the inter-donor consistency, with an inter-donor range of 0.406 cumulative population doublings after 18 days compared with 2.013 in FBS-containing medium. Identity and quality characteristics of the BM-hMSCs are also comparable between conditions in terms of colony-forming potential, osteogenic potential and expression of key genes during monolayer and post-harvest from microcarrier expansion. BM-hMSCs cultured on microcarriers in HPL-containing medium demonstrated a reduction in the initial lag phase for both BM-hMSC donors and an increased BM-hMSC yield after 6 days of culture to $1.20 \pm 0.17 \times 10^5$ and $1.02 \pm 0.005 \times 10^5$ cells/mL compared with $0.79 \pm 0.05 \times 10^5$ and $0.36 \pm 0.04 \times 10^5$ cells/mL in FBS-containing medium. *Conclusions.* This study has demonstrated that HPL, compared with FBS-containing medium, delivers increased growth and comparability across two BM-hMSC donors between monolayer and microcarrier culture, which will have key implications for process transfer during scale-up.

Key Words: *bioprocess, cell-based therapy, comparability, harvest, human platelet lysate, manufacture, mesenchymal stromal cell, microcarrier expansion, process development, process transfer*

Introduction

The growing field of regenerative medicine (RM) aims to treat unmet clinical indications such as cardiovascular disease and neurological disorders by restoring or maintaining cell or tissue function. Cell-based therapies form a large part of this RM industry and have the potential to transform health care. Human mesenchymal stromal cells (hMSCs) are of particular interest with clinical trials currently underway for multiple indications [1]. For the majority of these clinical indications, however, the *in vitro* expansion of cells is required to deliver an effective therapeutic dose without impacting the quality of the cell. Understanding and defining the quality attributes of hMSC therapies will be critical for their successful manufacture. This is proving difficult, however, because of their complex, multifaceted and poorly understood *in vivo* mechanisms of action [2].

At present, the majority of hMSC expansion takes place in static monolayers (T-flasks or multilayer flasks), which are suitable for the manufacture of the cell

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numbers required for early clinical development. However, it is widely recognized that these manual processes will not be sufficient to cost-effectively meet the needs for large-scale commercial production, where lot sizes will likely be on the order of trillions of cells [3]. For processes to drive toward the production of cost-effective therapies, they should be scalable, compliant with Good Manufacturing Practices and amenable to closed and automated process steps.

Microcarriers have been used to culture adherent cells such as BM-hMSCs in suspension in stirred bioreactors [4], allowing for process scale-up in which online monitoring and control systems can be used to deliver consistent and cost-effective BM-hMSC products [5]. Stirred tank bioreactors are currently employed for mammalian cell culture in biopharmaceutical production, and therefore their design and operation are well understood [6], with the potential to meet the expected manufacturing demands of large-scale BM-hMSC therapies. However, considerable work is required to demonstrate a satisfactory level of comparability between the traditional monolayer processes and these suspension-based systems in terms of cellular growth and quality.

A key aspect of these manufacturing processes is the culture medium in which the cells are to be expanded, which is typically supplemented with fetal bovine serum (FBS) [7]. In addition to lot-to-lot variability, there are further process constraints on the use of FBS, such as limited supply, spiraling costs [8], potential for pathogen transmission and immunological reactions against bovine antigens [9]. Human platelet lysate (HPL) has been proposed as a viable alternative in which blood platelets are lysed to release growth factors such as platelet-derived growth factor (PDGF), epidermal growth factor (EGF), transforming growth factor-β (TGF-β) and fibroblast growth factor (FGF), which then supplement the BM-hMSC growth medium [10]. This can be used as a patient specific supplement from their own blood plasma or pooled from multiple donors—for example, Stemulate manufactured by Cook Regentec. Furthermore, HPL has already been reported as a superior substitute to FBS for the *in vitro* expansion of BM-hMSCs [11] and has been shown to maintain the key cell characteristics and multipotent capacity [12]. Despite this, little work has been done to demonstrate the amenability of HPL to a scalable BM-hMSC manufacturing process, such as microcarrier suspension culture in stirred tank bioreactors.

Therefore, the aim of this study was to compare the relative performance of HPL to FBS for the monolayer expansion of BM-hMSCs and the amenability of HPL to transfer to a microcarrier-based suspension culture.

Methods

Monolayer culture

Human MSCs were isolated from bone-marrow aspirate purchased from Lonza obtained from two healthy donors with informed consent: BM-hMSC 1 (lot 071313B) and BM-hMSC 2 (lot 071281D). These two BM-hMSC donors were selected after pre-screening of five potential donors because of their differing growth and characteristics [13], representing the two extremes in terms of culture performance (±5.2 population doublings over 30 days of expansion). The local ethics committee approved the use of the samples for research. Cells from passage 1 were cryopreserved at a density of $1–2 \times 10^6$ cells/mL in a freeze medium containing 90% (v/v) FBS (Hyclone) and 10% (v/v) dimethylsulphoxide (Sigma-Aldrich). Cells were grown in T-flasks seeded at 5000 cells/cm^2 at 37°C in humidified air containing 5% CO_2. Dulbecco's Modified Eagles Medium (1 g/L glucose; Lonza) supplemented with 10% (v/v) FBS (Hyclone) or 10% (v/v) non-heparin requiring (PL-NH) Stemulate (Cook Regentec) and 2 mmol/mL UltraGlutamine (Lonza) was exchanged every 3 days. The FBS batch used in this study was selected from a number of tested FBS batches for its favorable growth performance, while retaining key BM-hMSC characteristics. On passage, the BM-hMSCs were washed with phosphate-buffered saline (PBS) without Ca^+ or Mg^+ and incubated for 4 min with trypsin (0.25%)/ethylenediaminetetraacetic acid (EDTA; Lonza) for FBS-based culture or TrypLE Express (Invitrogen) for PL-NH Stemulate-based culture. Dissociation reagents were inactivated by the addition of appropriate growth medium, and the cell suspension was centrifuged at 220*g* for 5 min. The supernatant was discarded and the remaining pellet was re-suspended in an appropriate volume of culture medium. For PL-NH Stemulate culture, BM-hMSCs underwent one adaptation passage in medium containing 10% (v/v) PL-NH Stemulate. Data represents four experimental repeats for each condition.

Spinner flask culture

The glass surfaces of 100-mL spinner flasks (diameter T = 60 mm; BellCo) with a magnetic, horizontal stirrer bar and a vertical paddle (diameter D = 50 mm) were siliconized with Sigmacoat (Sigma-Aldrich) according to the manufacturers' instructions. Solid, non-porous Plastic P-102L microcarriers (Solohill) at 500 cm^2/100 mL were prepared following the manufacturer's instructions. Microcarriers were preconditioned in 50 mL FBS or PL-NH Stemulate-containing growth medium for 1 h before BM-hMSC inoculation at 6000 cells/cm^2 and cultured in 100 mL of FBS-containing or PL-NH Stemulate-containing medium at 37°C in humidified

air containing 5% CO_2. A 50% medium exchange was performed every 3 days. After inoculation, the culture was static for 1 h, after which the culture was agitated constantly at the minimum rate for suspension (N_{JS}) found, experimentally, to be 30 rpm, with daily medium samples of 1 mL taken for analysis. Data represent four experimental repeats for each condition.

Analytical techniques

Measurement of glucose, lactate and ammonia concentration in spent medium was performed using a Cedex Bio-HT (Roche, Germany). Cell counting, mean cell diameter and viability (via acridine orange uptake and DAPI exclusion) was performed using a NucleoCounter NC-3000 automated mammalian cell counter (Chemometec). Microcarrier-based cell counts were obtained while the cells were still attached to microcarriers. The following parameters were obtained:

1. Specific growth rate:

$$\textit{Specific growth rate}, \quad \mu = \frac{\ln\left(C_x(t) \big/ C_x(0)\right)}{\Delta t},$$

where μ is the net specific growth rate (h^{-1}), $C_x(t)$ and $C_x(0)$ are the cell numbers at the end and start of the exponential growth phase, respectively and t is time (h).

2. Population doublings:

$$\textit{Population Doublings}, \quad P_d = \frac{1}{\log(2)} \cdot \log\left(\frac{C_x(t)}{C_x(0)}\right),$$

where P_d is the number of population doublings, $C_x(t)$ and $C_x(0)$ are the cell numbers at the end and start of the exponential growth phase, respectively.

3. Specific metabolite consumption/production rate

$$\textit{Specific metabolite flux}, \quad q_{met} = \left(\frac{\mu}{C_x(0)}\right) \cdot \left(\frac{C_{met}(t) - C_{met}(0)}{e^{\mu t} - 1}\right),$$

where q_{met} is the net specific metabolite consumption or production rate, μ is the specific growth rate (h^{-1}), $C_x(0)$ is the cell number at the end of the exponential growth phase, $C_{met}(t)$ and $C_{met}(0)$ are the metabolite concentrations at the end and start of the exponential growth phase, respectively, and t is time (h).

Microcarrier harvest

Human MSCs were harvested using a method that we developed previously [14,15]. Briefly, culture medium was removed from the spinner flask, and cells were washed twice with 100 mL Ca^{2+}- and Mg^{2+}-free PBS and dissociated with 50 mL of TrypLE Express (Stemulate culture) or trypsin (0.25%, w/v)/EDTA (FBS culture). After cell detachment from the microcarriers using a short period of high agitation at $5 \times N_{JS}$ (150 rpm), BM-hMSCs were separated from microcarriers using a 60-μm Steriflip filter (Millipore). The cell suspension was then centrifuged and re-suspended in the appropriate culture medium.

Quantitative osteogenesis assay

Osteogenesis was quantified by BM-hMSC collagen production using the Sircol Assay (Biocolour) following osteogenic differentiation. Collagen standards of acid-soluble collagen Type I at 0, 0.1, 0.2 and 0.4 g/L were used to quantify collagen production. BM-hMSCs were seeded at 10 000 cells/cm^2 in a well plate with the previously described cell culture medium; after 3 days, culture growth medium was exchanged to osteogenic medium (Irvine Scientific) and cultured for 9 days with a medium exchange taking place every 3 days. To quantify the collagen production cells were fixed with a solution of 5% acetic acid (v/v) (Sigma) and 9% formaldehyde (v/v) (Sigma) for 30 min at room temperature. The monolayer was washed and Sircol Dye Reagent (Biocolour) was added to each well for 30 min, removed and the cell monolayer was washed with Acid-Salt Wash Reagent (Biocolour). Alkali Reagent (Biocolour) was added to each well to release the collagen-bound Sircol Dye Reagent, and the resulting solution along with the collagen standard was quantified on a microplate reader (BMG Labtech) at an absorbance of 555 nm [13].

Colony-forming unit fibroblast efficiency

To assess the colony-forming unit fibroblast efficiency, BM-hMSCs were seeded in a T-flask at 10 cells/cm^2 and cultured with a medium exchange every 3–5 days. After 14-day culture, cells were washed with PBS and fixed in 4% formaldehyde (v/v) (Sigma) for 30 min. Colonies were stained with 1% crystal violet (Sigma) in 100% methanol (w/v) for 30 min. Stained colonies that were made up of more than 25 cells were recorded as colony-forming units [13,16].

RNA isolation

Cells were harvested for RNA isolation at the beginning, middle and at the end of the expansion process. Total RNA was collected using TriFast Reagent (Peqlab) according to the manufacturer's instructions. Potential genomic DNA contamination was removed by digestion with DNase I (Life Technologies, Germany) followed by reverse transcription at 50°C for 60 min using Superscript III (Life

Technologies) and 250 ng Oligo(dT)$_{18}$-primer (Life Technologies).

Quantitative real-time polymerase chain reaction analysis

Quantitative real-time polymerase chain reaction polymerase chain reaction was done with SYBR GreenER qPCR Supermix Universal (Life Technologies), additionally added 1× SybrGreenI (Life Technologies) and 0.2 µmol/mL primer each on the DNA engine Opticon2 (Bio-Rad) using these cycling conditions: Primary denaturation at 95°C for 3 min, followed by 35 cycles: 95°C for 30 s, 60°C for 30 s (36B4, p21, CCL2, VEGF)/55°C (Oct4) and 72°C for 30 s followed by fluorescence measurement. The following primers for cell markers were used: CCL2 (recruits monocytes, memory T cells, and dendritic cells to the sites of inflammation produced by either tissue injury or infection, NM_002982.3) (Fw) 5′-CCA AGG GCT CGC TCA GCC AGA TGC-3′, (Re) 5′-CGG AGT TTG GGT TTG CTT GTC CAGG-3′; p21 (regulates the cell cycle and mediates cellular senescence, NM_000389.4) (Fw) 5′-CCG CCT GCC TCC TCC CAA CT-3′, (Re) 5 ′-GAG GCC CGT GAG CGA TGG AA-3′, OCT4 (pluripotent marker associated with self-renewal of undifferentiated cells, NM_002701.4) (Fw) 5′-GAG GAG TCC CAG GAC ATC AA-3′, (Re) 5′-CAT CGG CCT GTG TAT ATC CC-3′ and VEGF (associated with vascularization and growth of blood vessels, NM_001171623.1) (Fw) 5′-GGAAGGAGCCTCCCTCAGGGTTTCG -3′, (Re) 5′- GCCGGAGTCTCGCCCTCCGG -3′. Serial dilutions of plasmid standards were used as positive controls and for quantification. Expression was normalized to the reference gene 36B4 (ribosomal protein large P0 RPLP0, NM_001002.3) (Fw) 5′-CTCGCTTCCTGGAGGGTGTCCGC -3′, (Re) 5′-CTCCACAGACAAGGCCAGGACTCG -3′ [16].

hMSC characterization

Immunophenotype analysis was performed by multiparameter flow cytometry before and after the BM-hMSC expansion process using a previously developed protocol [17]. Short tandem repeat analysis was completed by LGC Standards (UK) under their cell line authentication program. Morphology images were obtained using a light microscope (Nikon Eclipse TS-100).

The BM-hMSC differentiation was induced using PRIME-XV Differentiation Serum-Free Medium (Irvine Scientific) according to the manufacturer's instructions. After 21 days the differentiation media were removed, cells rinsed with PBS then fixed with 4% (v/v) paraformaldehyde at room temperature. Adipocytes were stained with 1% (w/v) oil red O (Sigma-Aldrich) in isopropanol at room temperature and rinsed with distilled water. Osteoblasts were incubated with 2.5% (v/v) silver nitrate (Sigma-Aldrich) under ultraviolet light (30-min exposure), rinsed with distilled water and stained with fast violet solution (Sigma-Aldrich) containing 4% (v/v) naphthol AS-MX phosphate alkaline (Sigma-Aldrich) for 45 min at room temperature in the dark. Chondrocytes were stained with 1% (w/v) Alcian blue (Sigma-Aldrich) in 0.1 mol/L hydrochloric acid (Sigma-Aldrich). After 30-min incubation, cells were rinsed three times with 0.1 mol/L HCl. After staining, differentiated cells were visualized under a light microscope (Nikon Eclipse TS-100) [13].

Statistical analysis

Results were deemed to be significant if $P < 0.05$ using a two-tailed Student's *t*-test assuming unequal variances.

Results

Monolayer expansion of BM-hMSCs

The successful translation of many BM-hMSC therapies will require processes that enable cell expansion because the therapeutic dose is likely to exceed the number of harvested cells. It can be seen from Figure 1(A,B) that BM-hMSCs expanded in HPL have significantly higher ($P < 0.05$) growth rate for both donors compared with FBS. This is most apparent over the first three passages (18 days culture) before the cells expanded in HPL undergo a senescent phase at approximately 10 cumulative population doublings in the monolayer expansion process. It can be seen from Figure 1C that this movement into the senescent phase is associated with an increase in mean cell diameter through expansion, which has been reported previously [13,16] and can potentially be used as a process metric to indicate the onset of senescence during a BM-hMSC manufacturing process. It will be important to minimize this level of senescence during the manufacturing process because it is likely to have a detrimental impact on the quality and quantity of the final product. This action is particularly important in the HPL-based process, which encounters this senescent phase much earlier during expansion.

It is important that the BM-hMSCs expanded during this process also maintain their identity, as defined by the International Society for Cellular Therapy minimum criteria. Short tandem repeat analysis has confirmed the genotype of both BM-hMSC lines at the end of the expansion process in both conditions (supplementary Figure S1), although previous studies have demonstrated that changes to BM-hMSC karyotype does not affect their *in vivo*

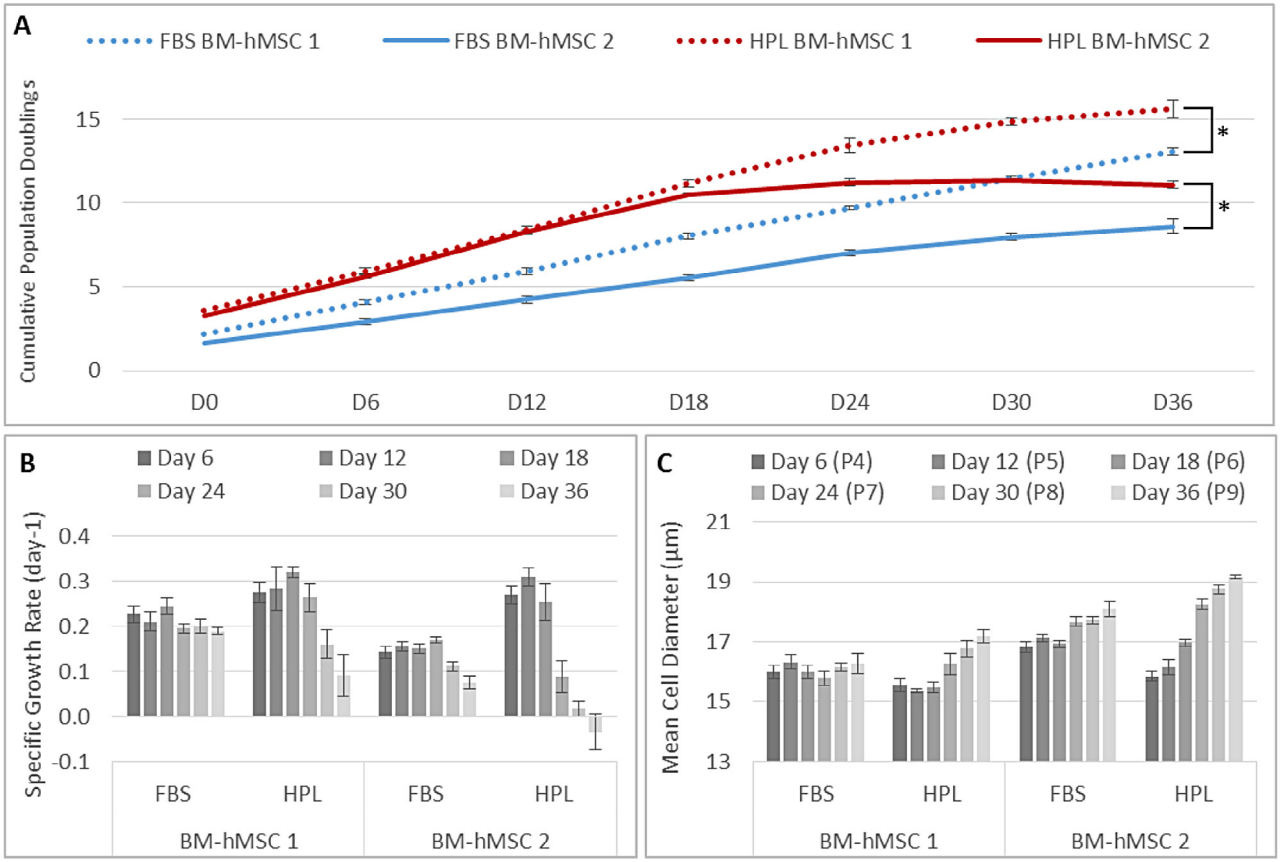

Figure 1. Growth rates of the two BM-hMSC lines over 36 days of expansion. Showing (A) cumulative population doublings showing increased growth rate in HPL ($P < 0.05$), (B) specific growth rate of hMSCs in FBS and HPL and (C) mean cell diameter throughout culture. Data shows mean ± SD (n = 4).

characteristics [18]. Tri-lineage differentiation potential has also been maintained (supplementary Figure S2) and immunophenotype has been confirmed by multiparameter flow cytometry (supplementary Figure S3). In addition to BM-hMSC identity, it is also important to consider the effect of the expansion process on the quality attributes of the BM-hMSCs because these will be more relevant to the function of the therapy.

The presence of key BM-hMSC genes through the expansion process is also important because they are likely to play a key role in the *in vivo* function of the cell. Figure 4 shows the RNA expression of the donor BM-hMSC lines in FBS- and HPL-containing medium against population doubling level, with maintained expression of P21 and CCL2. The CCL2 gene has been implicated in the recruitment of T cells, monocytes and dendritic cells to sites of inflammation [36] and is therefore an important gene of which to retain expression for clinical indications that require a level of immune modulation. Figure 4B shows the relative expression of P21, a gene relating to cellular aging and senescence, which has not increased significantly throughout the expansion process in HPL- nor FBS-based expansion.

Microcarrier expansion of BM-hMSCs

The growth rates of BM-hMSCs will also play a part in reducing costs because accommodating BM-hMSCs with a lower expansion potential will reduce annual production rates [13,19]. Figure 6A shows the growth rate of the two BM-hMSC donors over 6 days in culture in both FBS- and HPL-containing media. The growth rates of the two donor BM-hMSCs in the HPL-based medium is significantly higher ($P < 0.01$) than the FBS-containing medium for both of the BM-hMSC lines, in particular BM-hMSC 2 showed extremely low growth rates in FBS with 0.44 ± 0.18 population doublings over the 6-day growth period. This increase in growth rate for BM-hMSCs during microcarrier culture has also been demonstrated at 5% HPL compared with 10% FBS [20]. BM-hMSCs cultured on microcarriers in HPL-containing medium demonstrated an increased BM-hMSC yield after 6 days of culture to $1.20 \pm 0.17 \times 10^5$

and $1.02 \pm 0.005 \times 10^5$ cells/mL compared with $0.79 \pm 0.05 \times 10^5$ and $0.36 \pm 0.04 \times 10^5$ cells/mL in FBS-containing medium. Despite the improved yield in HPL-containing medium, these cell yields still need to be further increased to produce 10^{12} BM-hMSCs per batch and thus to be economical even at the commercial scale. It is also likely that increasing the functionality of the BM-hMSC product will play a role in reducing costs, and therefore it will be important to assess the effect of different culture conditions on product functionality.

The net metabolite flux of the cells in microcarrier culture has been measured for BM-hMSC 1 and BM-hMSC 2. Figure 7A shows the glucose consumption of BM-hMSCs in FBS and HPL culture, which was between 11.13 and 15.32 pmol/cell/day with no significant difference measured across these conditions. The lactate production rate is shown in Figure 7B, which again shows a similar level of production between FBS and HPL. This suggests that the BM-hMSCs in both conditions are using similar metabolic pathways, as confirmed by the yield of lactate from glucose (Figure 7D). This is in contrast to previous studies using serum-free medium, which demonstrated that BM-hMSCs tend to favor the relative production of ammonia over lactate during microcarrier culture under serum-free conditions [21]. There was, however, a reduction in the production of ammonia for BM-hMSC 1 in HPL to 1.20 ± 0.03 pmol/cell/day, suggesting altered amino acid use, which may be related to the need for precursors (e.g., glutamine and asparagine) supporting purine and pyrimidine biosynthesis [22].

Discussion

Monolayer expansion of BM-hMSCs

Increasing the manufacturing consistency between donors will be important for increasing the cost-effectiveness of cell-based therapy processes and will support further development and future scale-up [13,16]. It can be seen from Figure 1A that the growth rates between the two donor BM-hMSCs in HPL are not only higher but more consistent. This difference in behavior can be explained in part by the change in morphology of the BM-hMSCs in HPL (Figure 2), which is similar for both of the BM-hMSC donors. In contrast, the same donor cells expanded in FBS-containing medium have a markedly different morphology.

Previous studies have highlighted the importance of minimizing the expansion ratio of BM-hMSCs because their therapeutic potential decreases as the number of cumulative population doublings increases [23,24]. Figure 3A shows the effect of the increasing number of population doublings on the colony-forming potential of the BM-hMSCs, with a general decline as the number of population doublings increases. This decline has been highlighted by others [25] and will have implications for off-the-shelf manufacturing processes requiring a large number of cells. Declining quality through expansion will result in the

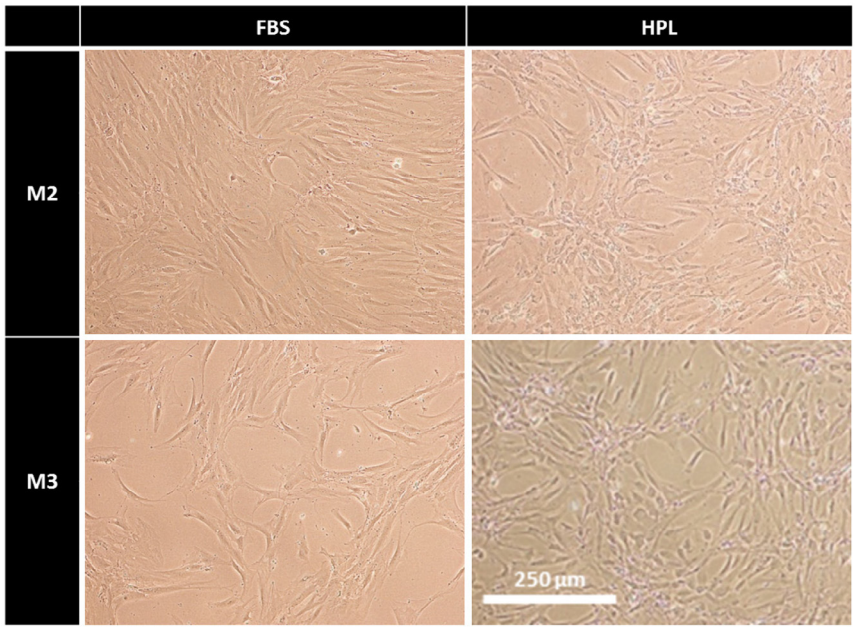

Figure 2. Phase-contrast images of the two BM-hMSC lines showing the increased consistency in morphology between the cell lines under HPL expansion compared with FBS.

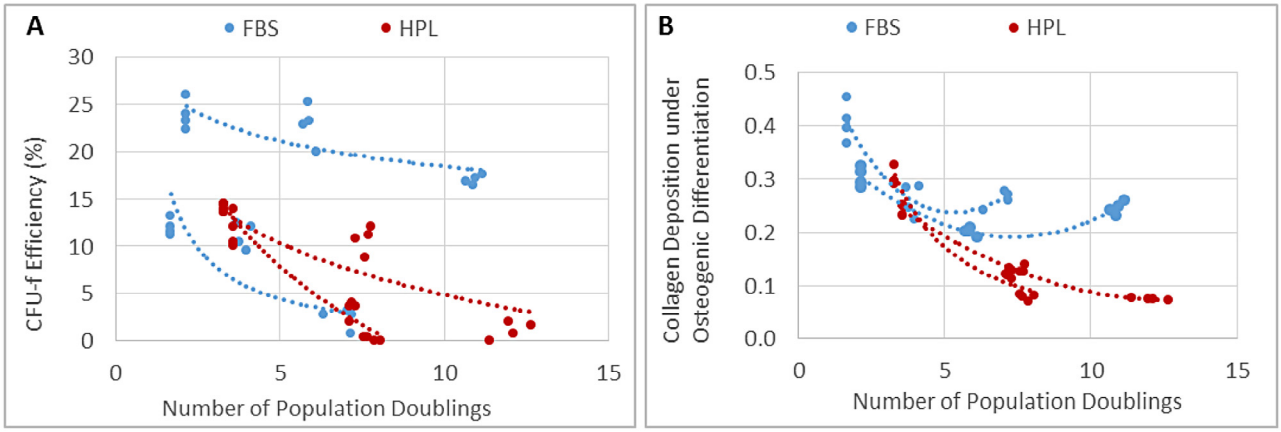

Figure 3. Colony-forming efficiency and osteogenic potential of two BM-hMSC lines against number of population doublings throughout the expansion process. Showing (A) colony-forming efficiency and (B) collagen production under osteogenic conditions demonstrating the decrease through culture. CFU-f, colony-forming unit fibroblast.

use of multiple donor samples for the expansion process to reduce the overall number of population doublings and maintain product quality. The reduced colony-forming potential is particularly apparent for HPL-based culture, which should be further investigated before process transfer to ensure it is not having a detrimental impact on the product quality for a particular clinical indication, although it may not necessarily be a relevant marker for all applications.

This trend is also presented in Figure 3B, which shows that the osteogenic potential of the BM-hMSCs decreases as the number of population doublings increases. Such results are further supported by *in vivo* clinical data [26] for these indications as well as for additional indications outside of the BM-hMSC niche [27]. It is important to assess the impact of the expansion process on quality attributes early in development because it will determine the maximum allowable expansion ratio of the product for each particular indication, which will in turn influence the overall cost and scale of the therapy.

Despite the maintenance of CCL2 and P21 during expansion, the RNA expression of Oct4 and VEGF has shown a slight decrease with increasing population doublings. Oct4 is a marker of pluripotency, mainly associated with embryonic stem cells, but has previously shown expression in BM-hMSCs [37] and will be important for cell-based products that require cellular differentiation. VEGF has been shown to be a highly important gene in the promotion of angiogenesis by BM-hMSCs [38], which will be particularly important for cardiac repair, a key target for a number of BM-hMSC-based therapies. Despite a higher relative expression of VEGF in BM-hMSCs cultured in HPL compared with FBS, there is a decrease in its expression as the number of population doublings increases, which should be further investigated if the BM-hMSC product requires a high level of cumulative population doublings and is to be used for clinical indications requiring some level of angiogenesis.

Measuring cell metabolite flux during the expansion process is likely to form the basis of many online BM-hMSC monitoring systems during manufacture, as it has been for many traditional bioprocesses [28]. The implementation of online monitoring processes for BM-hMSC production will allow for control systems to be put in place, which will be essential to reduce variation in product attributes and increase process consistency. The development of these control systems for BM-hMSC manufacturing processes is currently at an early stage, and a better understanding of the cell must be gained before models can be developed to simulate the effect of changing metabolite levels on cellular growth and function. Figure 5 shows the relative flux of glucose, lactate and ammonium throughout expansion, which increases as the BM-hMSCs move toward senescence. This change highlights the potential value of introducing an online monitoring system for metabolite flux because increasing cell size and per-cell metabolite flux could be used as an early indication of this senescence, and therefore process interventions (such as cell harvest) could be made to ensure that senescence does not affect the final product.

Microcarrier expansion of BM-hMSCs

For numerous clinical indications, BM-hMSCs will need to be manufactured on a large scale (on the order of 10^{12} cells per batch) to reduce the cost of production and to meet the market need for the treatment. Microcarriers have previously been demonstrated to support the proliferation and harvest of BM-hMSCs in suspension [4,29,30] and therefore have the

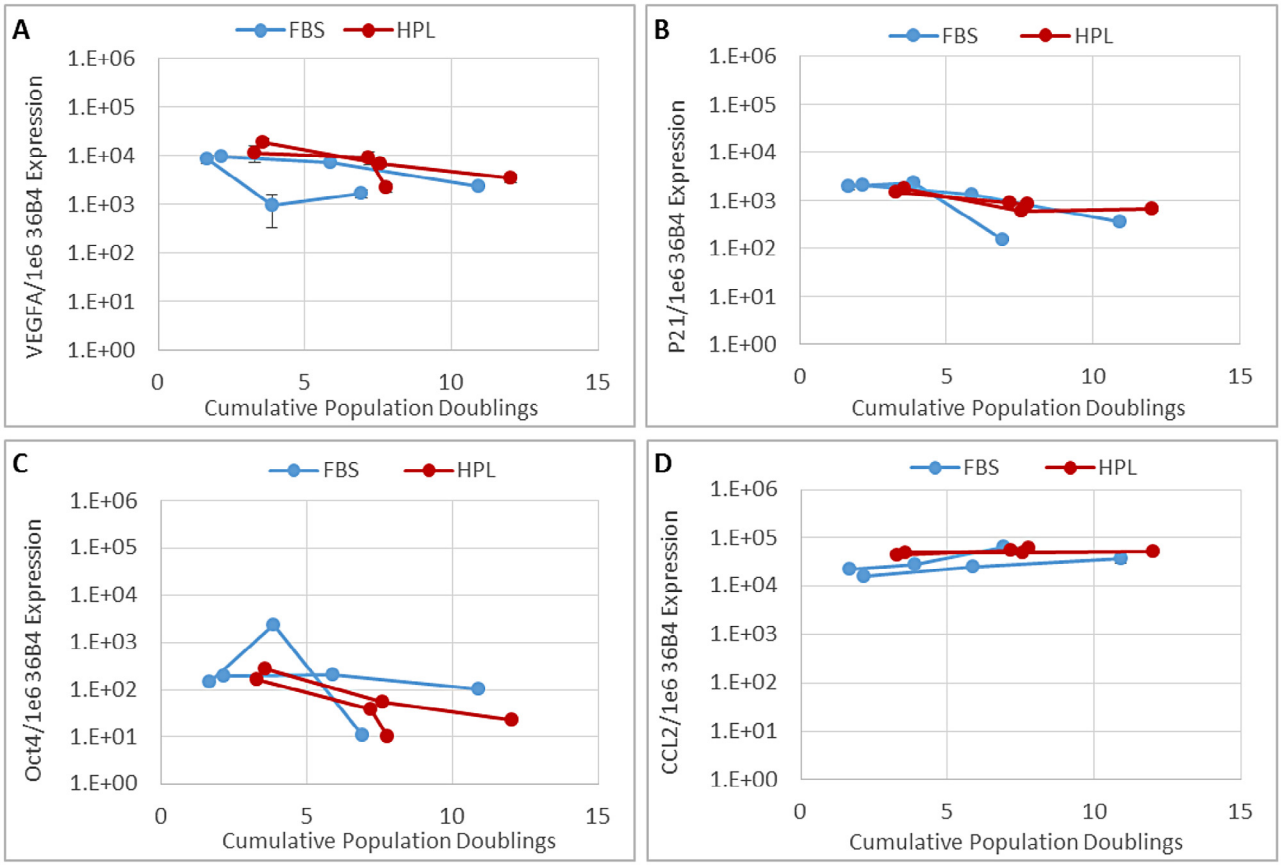

Figure 4. Quantitative real-time polymerase chain reaction analysis showing RNA expression of four hMSC genes for the two BM-hMSC lines in FBS and HPL expansion. Showing maintained expression of VEGFA (A), P21 (B) and Oct4 (C) and CCL2 (D). cDNA is normalized to housekeeping gene 36B4.

potential to be operated up to and beyond the thousand-liter scale.

The increased consistency between donors gives significant advantages to the manufacturing process because increased production rates and reduced batch failure rates are likely to reduce the overall cost of the product. It can be seen from Figure 6B that the lag phase experienced by BM-hMSCs in FBS culture between days 0 and 3 is not present when the cells are cultured in HPL, which is the likely cause of the difference in growth rates over the 6-day period. The reason for this may be due to an increase in the attachment rate of the cells, which in turn shortens the lag phase prior to cell division taking place. This link between attachment efficiency and cell growth is well established [31] and the level of relevant attachment proteins present in HPL is typically higher than in FBS, which will contribute to this effect [32]. The attachment of the BM-hMSCs to the culture surface is particularly important for suspension culture, where the cells and microcarriers are constantly agitated throughout the culture period.

The post-expansion detachment and separation of the BM-hMSCs from the microcarriers is of critical importance for a scalable production process because cellular attributes must be maintained throughout the process [14,15]. The same harvest protocol adopted by Nienow et al. [14,15] was modified for this study by replacing trypsin-EDTA with TrypLE Express for the HPL culture to ensure the process was animal-component free. The post-harvest viability from both donor BM-hMSC lines in FBS and HPL was >95%, demonstrating that this harvest process did not have a detrimental impact on the membrane integrity of the cells. It is important that BM-hMSC characteristics are also maintained after this detachment and separation process from the microcarriers. Figure 8 shows the effect of the total microcarrier process on BM-hMSC attributes compared with pre-expansion for colony forming efficiency (Figure 8A), osteogenic potential (Figure 8B), specific outgrowth rate (Figure 8C) and mean cell diameter (Figure 8D). The colony-forming efficiency of the BM-hMSCs following the microcarrier expansion and harvest process generally saw an increase, with a significant increase for BM-hMSC 1 in HPL ($P < 0.05$) compared with pre-expansion. Similarly, the specific outgrowth rate and mean cell diameter have been maintained

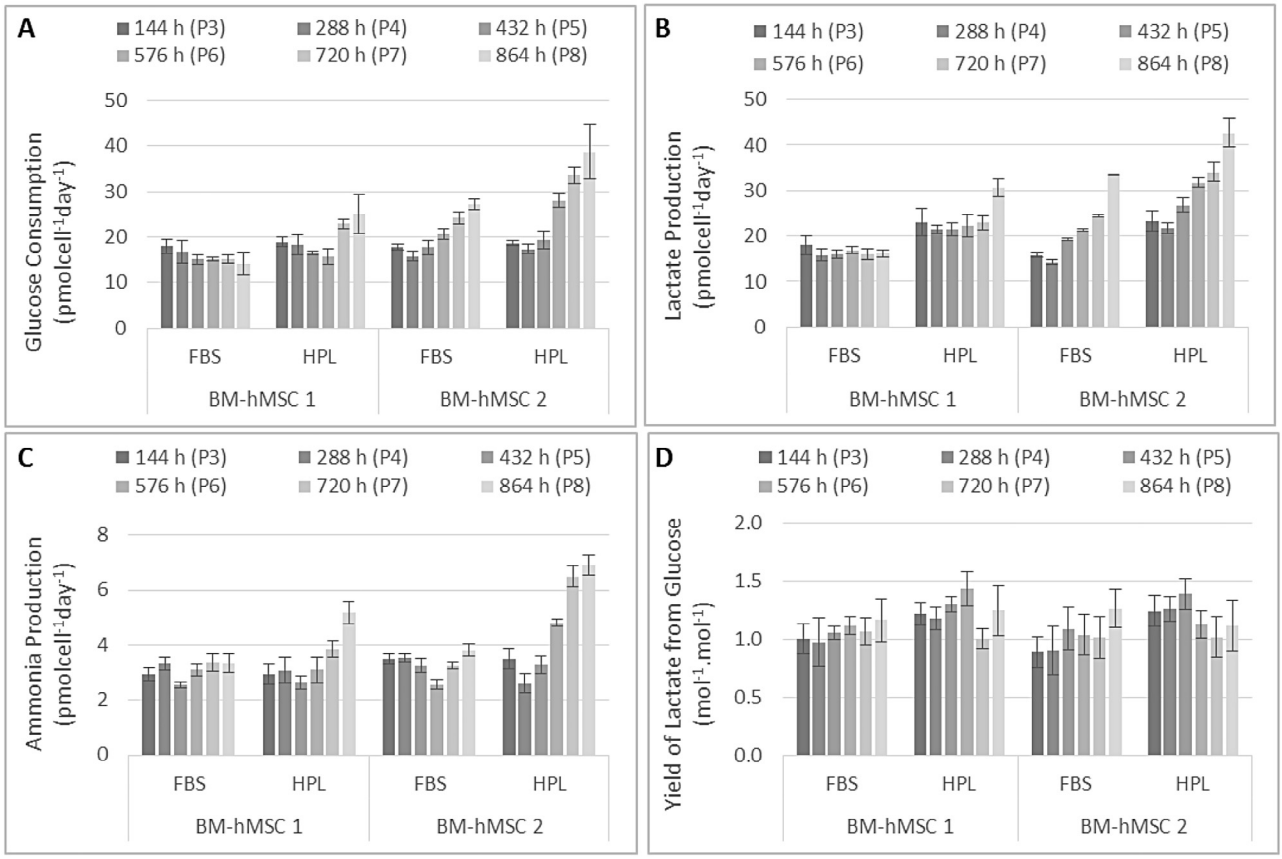

Figure 5. Per-cell metabolite flux of two BM-hMSC lines over 36 days of monolayer expansion. Showing (A) increasing per-cell glucose consumption rate through culture, (B) increasing per cell lactate production rate through culture, (C) increasing per-cell ammonia production rate through culture and (D) yield of lactate from glucose through culture. Data are mean ± SD (n = 4).

post-harvest, demonstrating that the microcarrier process has not affected these BM-hMSC characteristics. In contrast, the osteogenic potential of the BM-hMSCs decreased for all conditions ($P < 0.01$), which should be further investigated if the BM-hMSCs are to be used for clinical indications relating to the production of collagen. There is the potential however, for this type of clinical indication, that biodegradable microcarriers could be directly implanted as a cell-scaffold construct to support the regeneration of bone tissue [33], removing the need for cell harvest altogether.

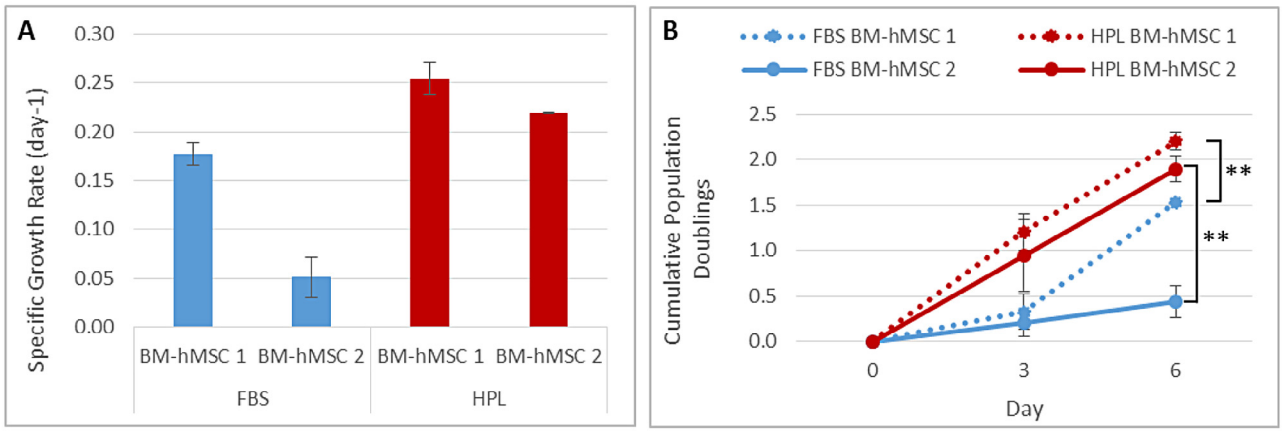

Figure 6. Growth rates of hMSCs cultured on microcarriers in FBS-containing medium and HPL-containing medium for 6 days showing increased growth rates in HPL ($P < 0.01$). (A) Specific growth rate and (B) cumulative population doublings. Data are mean ± SD (n = 4).

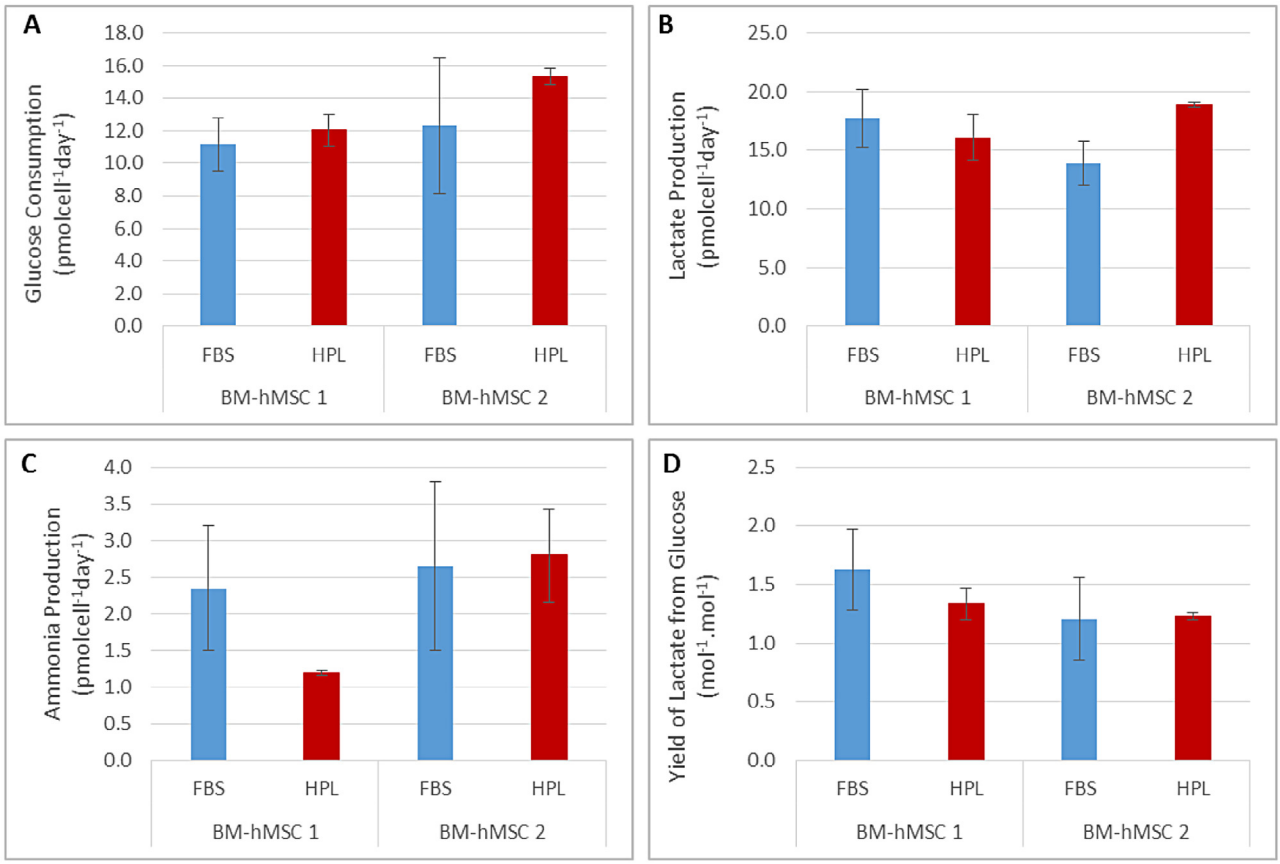

Figure 7. Per-cell metabolite flux of two BM-hMSC lines over 6 days of microcarrier expansion. Showing (A) per-cell glucose consumption rate, (B) per-cell lactate production rate, (C) per-cell ammonia production rate and (D) yield of lactate from glucose. Data are mean ± SD (n = 4).

This comparison of performance demonstrates that HPL is a viable alternative to FBS for the microcarrier culture of BM-hMSCs and has the potential to be taken forward to support further process development and scale-up. The increased consistency in growth between donors in HPL will also benefit the development of both patient specific and off-the-shelf BM-hMSC therapies, allowing for increased production rates and shorter processing times. That said, more work is required to integrate further quality and functionality assays into the microcarrier process. This is critical to ensure comparability as the bioprocess is developed further and scaled-up.

Comparison of monolayer and microcarrier culture for process transfer

One of the key challenges in the successful development of scalable cell therapy manufacturing processes is in the process transfer away from traditional manual monolayer techniques. The decision of when in the development cycle to complete these bridging studies and move toward a scalable process should be carefully considered, and many companies have incurred spiraling costs as a result of waiting until the end of clinical development before commercial production can commence. The advantages of automated and closed processing systems in driving scalable production by reducing costs should not be underestimated [34,35], with suspension-based systems being a lead candidate because they are routinely operated in this manner. This process transfer from monolayer to microcarrier culture will aim to assess the impact of the new process on the product attributes to ensure they are comparable between the processes, avoiding the need to repeat clinical work, which will require significant time and capital.

Obtaining similar growth rates of the BM-hMSCs from the monolayer and microcarrier processes is also important because this will affect the number of BM-hMSCs that can be manufactured per unit time, which will reduce the number of batches produced per year. Table I shows the comparison of the monolayer and microcarrier process for BM-hMSC 1 and 2 in FBS- and HPL-based culture. The growth rate of BM-hMSCs in FBS-based culture showed a significant reduction from monolayer to microcarrier culture for both BM-hMSC lines ($P < 0.05$ and $P < 0.01$), which

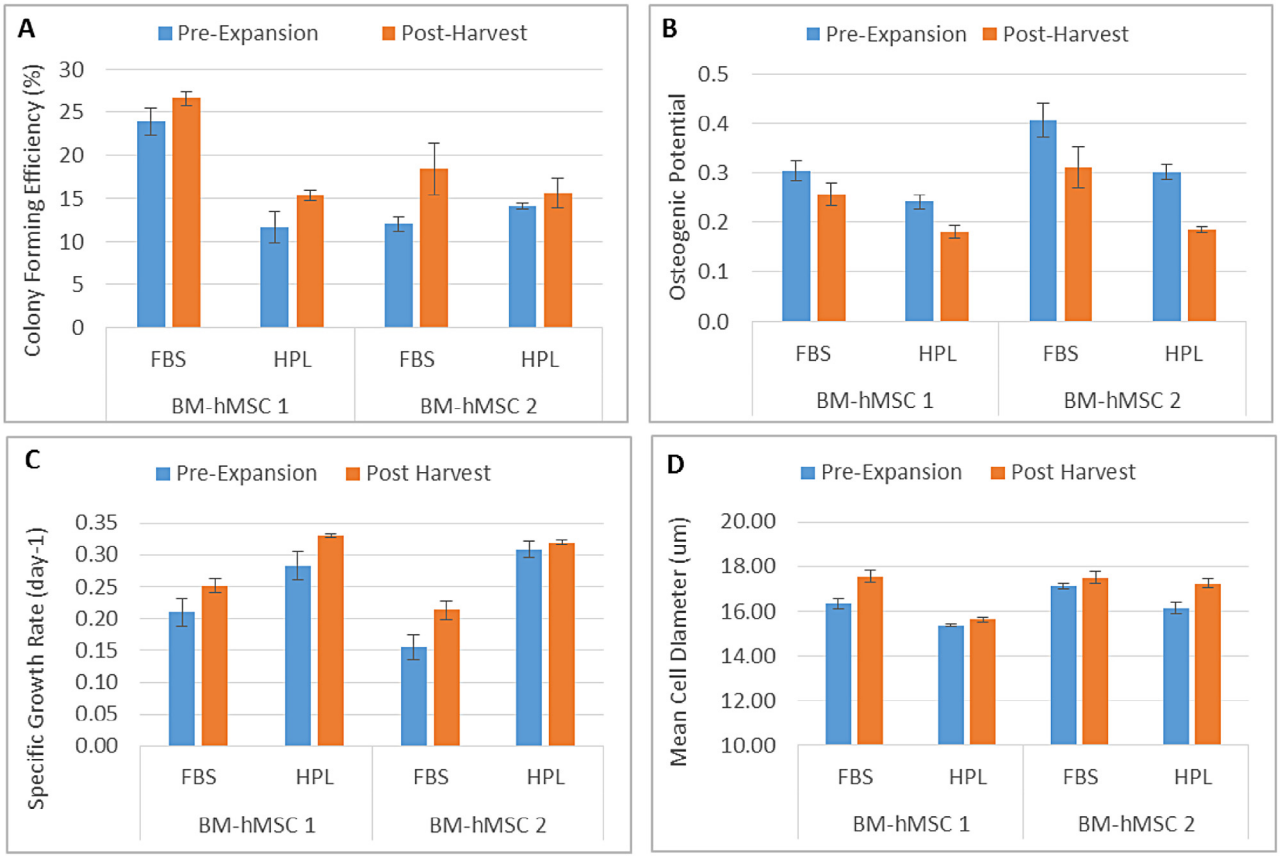

Figure 8. Post-harvest hMSC quality compared with pre-expansion demonstrating retention of key attributes, showing (A) colony-forming efficiency, (B) osteogenic potential, (C) specific growth rate and (D) mean cell diameter. Data are mean ± SD (n = 4).

is particularly apparent for BM-hMSC 2, with a reduction from 0.14 ± 0.02 to 0.05 ± 0.02 day. This reduction would create challenges during process scale-up within a suspension-based system and would necessitate careful selection of donor material for master cell banks to ensure it is amenable to this potential change in process conditions from static to agitated culture. It is likely that this widening of the gap in growth rates between the static monolayer process and agitated microcarrier culture is due to the efficiency of cell attachment, which becomes more important within the agitated environment.

In contrast to FBS-based culture, the use of HPL proved more effective in supporting the transfer of the process from static to agitated conditions with only a slight reduction in the growth rate of BM-hMSC 2 and no significant difference between the monolayer and microcarrier growth rate of BM-hMSC 1. This consistency demonstrates the potential of HPL to be used as a medium supplement within monolayer culture as well as for suspension culture of BM-hMSCs. Table I also shows that the colony-forming efficiency, mean cell diameter and outgrowth rate have not been reduced for the microcarrier process, which will be important during process transfer and scale-up toward commercial production. It is also evident from Table I that the yield of lactate from glucose is more similar when comparing monolayer and microcarrier culture in HPL with FBS, which suggests a maintenance of the glycolytic pathway during microcarrier culture [36]. All this means that HPL represents a beneficial alternative to FBS for monolayer expansion processes and further process transfer into suspension-based systems. This improvement has implications for both patient-specific and off-the-shelf BM-hMSC therapies in terms of increasing yield and reducing costs of therapies that are currently in development.

The development of serum-free microcarrier expansion processes will also be important in driving cost-effective BM-hMSC products [21,37]. However, serum-free production may not be amenable to every product because process transfer into suspension without the use of serum may not support the expansion of all cell types, with the time and cost associated with demonstrating process comparability for such a substantial process change becoming prohibitive as the process is scaled [38]. It is important that these competing factors are evaluated at an early stage to avoid unnecessary and costly process changes during clinical development.

Table I. Comparison of monolayer and microcarrier culture of hMSCs in FBS- and HPL-containing medium showing the improved process transfer under HPL.

	Growth rate (day^{-1})		Mean cell diameter (μm)		CFU efficiency (%)		Osteogenic potential		Outgrowth rate (day^{-1})		Yield of lactate from glucose (mol/mol)	
	ML	MC	ML	MC	ML	MC	ML	MC	ML	MC	ML	MC
BM-hMSC 1												
HPL	0.27 ± 0.02	0.25 ± 0.02	15.53 ± 0.06	15.47 ± 0.38	11.60 ± 1.82*	15.33 ± 0.61*	0.24 ± 0.01**	0.18 ± 0.01**	0.28 ± 0.05	0.33 ± 0.01	1.22 ± 0.10	1.34 ± 0.14
FBS	0.23 ± 0.02*	0.18 ± 0.01*	16.33 ± 0.22	14.93 ± 1.27	23.90 ± 1.54	26.60 ± 0.85	0.30 ± 0.02**	0.26 ± 0.02**	0.21 ± 0.02*	0.25 ± 0.01*	1.00 ± 0.13*	1.63 ± 0.35*
BM-hMSC 2												
HPL	0.27 ± 0.01*	0.22 ± 0.01*	16.15 ± 0.25*	15.53 ± 0.08*	14.10 ± 0.38	15.60 ± 1.74	0.30 ± 0.02**	0.19 ± 0.01**	0.31 ± 0.01	0.32 ± 0.01	1.24 ± 0.02	1.23 ± 0.03
FBS	0.14 ± 0.02**	0.05 ± 0.02**	17.13 ± 0.46**	15.23 ± 0.46**	12.00 ± 0.86	18.40 ± 3.02	0.41 ± 0.03**	0.31 ± 0.04**	0.16 ± 0.02*	0.21 ± 0.01**	0.89 ± 0.13	1.20 ± 0.35

Statistics show significance between monolayer and microcarrier condition.
CFU, colony-forming unit; MC, monocarrier; ML, monolayer.
*$P < 0.05$; **$P < 0.01$.

Conclusions

This study has demonstrated that pooled HPL represents a viable alternative to a pre-selected batch of FBS for the monolayer and microcarrier culture of BM-hMSCs. The maintenance of BM-hMSC growth potential between monolayer and microcarrier expansion in HPL-based medium offers significant advantages for process transfer during clinical development, which will be important for demonstrating comparability and reducing associated development costs.

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Appendix: Supplementary material

Supplementary data to this article can be found online at doi:10.1016/j.jcyt.2016.01.007.