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Serum-free process development: improving the yield and consistency of human mesenchymal stromal cell production

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Abstract

Background aims. The cost-effective production of human mesenchymal stromal cells (hMSCs) for off-the-shelf and patient specific therapies will require an increasing focus on improving product yield and driving manufacturing consistency. *Methods.* Bone marrow–derived hMSCs (BM-hMSCs) from two donors were expanded for 36 days in monolayer with medium supplemented with either fetal bovine serum (FBS) or PRIME-XV serum-free medium (SFM). Cells were assessed throughout culture for proliferation, mean cell diameter, colony-forming potential, osteogenic potential, gene expression and metabolites. *Results.* Expansion of BM-hMSCs in PRIME-XV SFM resulted in a significantly higher growth rate ($P < 0.001$) and increased consistency between donors compared with FBS-based culture. FBS-based culture showed an inter-batch production range of 0.9 and 5 days per dose compared with 0.5 and 0.6 days in SFM for each BM-hMSC donor line. The consistency between donors was also improved by the use of PRIME-XV SFM, with a production range of 0.9 days compared with 19.4 days in FBS-based culture. Mean cell diameter has also been demonstrated as a process metric for BM-hMSC growth rate and senescence through a correlation ($R^2 = 0.8705$) across all conditions. PRIME-XV SFM has also shown increased consistency in BM-hMSC characteristics such as per cell metabolite utilization, *in vitro* colony-forming potential and osteogenic potential despite the higher number of population doublings. *Conclusions.* We have increased the yield and consistency of BM-hMSC expansion between donors, demonstrating a level of control over the product, which has the potential to increase the cost-effectiveness and reduce the risk in these manufacturing processes.

Key Words: *cell-based therapy, comparability, consistency, human mesenchymal stromal cell, manufacturing, regenerative medicine, serum-free, yield*

Introduction

The successful development of cell-based therapies has the potential to address a number of currently unmet clinical indications and to improve patient care across the world. Growing interest in this emerging field is evident by the large number of recent acquisitions of cell-based therapy companies by larger biopharmaceutical multinationals; for example, FUJIFILM Holdings Corporation (TSE: 4901) recently acquired Cellular Dynamics International (NASDAQ: ICEL), a developer and manufacturer of induced pluripotent stem cells. However, despite the progress, there are a number of challenges that remain before cell-based therapies can be incorporated into routine clinical practice and their full potential realized.

Human mesenchymal stromal cells (hMSCs) have demonstrated the potential to target a number of these currently unmet conditions, with clinical trials currently underway for indications such as acute myocardial infarction, stroke and a host of inflammatory and immune disorders [1]. For the majority of these clinical indications, however, the *in vitro* expansion of cells is required to deliver an effective therapeutic dose. The intention of this expansion step is to manufacture a sufficient number

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of cells to deliver therapeutic benefit without having a detrimental impact on the quality of the cell at decreasing production costs. Understanding and defining the quality attributes of hMSC therapies will be critical for their successful manufacture. This is proving difficult, however, owing to their complex, multifaceted and poorly understood *in vivo* mechanism of action [2].

Cell-based therapies can be broadly divided into two categories: patient-specific therapies (autologous) and off-the-shelf therapies (allogeneic). Traditional biopharmaceutical manufacture is predominantly focused on universal treatments in which multiple patients can be treated from a single batch. The manufacture of patient-specific therapies, however, will require the careful consideration of regulatory challenges as well as the distribution and delivery of a safe, effective and affordable cell-based therapy [3]. This also introduces a range of additional challenges, not least of all how a cell therapy manufacturing process can be developed to consistently manufacture products from multiple donors [4]. This will also be necessary for off-the-shelf products because cellular senescence will limit their expansion potential [5]. The key difference between off-the-shelf and patient-specific therapies, however, is that a donor selection process can be used for off-the-shelf products to select donor cell lines that are similar on the basis of expansion potential and desired quality attributes.

A crucial factor determining the economic success of off-the-shelf cell-based therapies in terms of affordability probably will hinge on whether the patient receiving the hMSC therapy will require immunosuppressive medication, increasing the overall lifetime cost of the treatment, although most clinical trials do not currently use them [6]. It has been demonstrated previously [7,8] that the use of serum during cell culture processes can lead to an undesired increase in immune response *in vivo*, and therefore the use of serum-free alternatives has the potential to reduce the requirement for post-infusion immunosuppressive medication. The development of defined medium formulations for specific cell-based therapies can also use this type of clinical output as a basis for their development. These long-term considerations for hMSC product manufacture and delivery will be important to drive the development of cost-effective and reimbursable therapies, which has proved difficult to date.

Achieving the consistent manufacture of medicinal products is a key requirement for regulatory approval and begins with assessing and reducing process variation when possible [9]. Driving a consistent process will demonstrate a state of control over the product and provides a foundation for comparability, whereby process changes during clinical development can be validated and allows for the product to be manufactured at multiple sites. A key aspect of reducing variation in the process will be reducing and eventually eliminating the use of fetal bovine serum (FBS) from the cell culture medium [10]. In addition to lot-to-lot variability, there are further process constraints on the use of FBS such as limited supply [11], spiraling cost, potential for pathogen transmission, increased risk of recipient immune reaction [12] and reduced scope for process optimization. All of these considerations mean that moving toward a serum-free process would be beneficial in achieving scalable, tunable and consistent hMSC manufacturing processes. In addition, serum-free culture has been shown to be amenable to scalable expansion technology such as micro-carriers and stirred bioreactors, producing higher hMSC yields per time unit than serum-based processes, which will be important for driving down the production cost of hMSC therapies [13,14] and is a key focus for our group. That said, the current cost of serum-free medium (SFM) for research is generally higher than serum-based medium; however, as the demand increases and higher yield processes can be developed, these costs probably will be reduced over time.

Considering the innate biological variability that exists between donors and the importance of ensuring a consistent manufacturing process, driving this philosophy into process development at an early stage is critical. Therefore, the aim of this study was to demonstrate how the development of a serum-free expansion process can drive increased consistency and yield of hMSC manufacture between donors and the benefits that this can bring as the process scale increases.

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). The local ethics committee approved the use of the sample 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% (vol/vol) FBS (Hyclone) and 10% (vol/vol) dimethyl sulfoxide (Sigma-Aldrich). For serum-free experiments, hMSCs cryopreserved in serum underwent one adaptation passage in SFM. Cells were grown in T-flasks seeded at 5000 cells/cm^2 at 37°C in humidified air containing 5% CO_2. For serum-based culture,

Dulbecco's modified Eagle's medium (DMEM, 1 g/L glucose; Lonza) supplemented with 10% (vol/vol) FBS (Hyclone) and 2 mmol/L UltraGlutamine (Lonza) was exchanged every 3 days. For serum-free culture, the growth surface of T-flasks was coated with 0.4 μg/cm^2 PRIME-XV human fibronectin (Irvine Scientific) and cultured in PRIME-XV SFM (Irvine Scientific) according to the manufacturer's instructions. On passage, the hMSCs were washed with phosphate-buffered saline (PBS) without Ca^+ or Mg^+ and incubated for 4 min with trypsin (0.25 %)/ethylene diamine tetra-acetic acid (Lonza) for serum-based culture or TrypLE Express (Invitrogen) for serum-free 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.

Analytical techniques

Analysis of glucose and lactate concentrations in the spent medium was performed with the use of a Cedex Bio-HT (Roche). Cell counting, mean cell diameter and viability (by use of acridine orange uptake and 4′-6-diamidino-2-phenylindole exclusion) was performed with the use of a Nucleo-Counter NC-3000 automated mammalian cell counter (Chemometec). The following parameters were obtained:

Specific growth rate

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

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).

Population doublings

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

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

Specific metabolite consumption/production rate

$$\textit{Specific metabolite flux},$$

$$q_{met} = \left(\frac{\mu}{C_x(0)}\right) \cdot \left(\frac{C_{met}(t) - C_{met}(0)}{e^{\mu t} - 1}\right)$$

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).

Quantitative osteogenesis assay

Osteogenesis was quantified by means of hMSC collagen production with the use of the Sircol assay (Biocolour) after 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 the collagen production. Bone marrow–derived hMSCs (BM-hMSCs) were seeded at 10,000 cells/cm^2 in a well plate with 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 (vol/vol) (Sigma) and 9% formaldehyde (vol/vol) (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.

Colony-forming unit fibroblast efficiency

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

RNA isolation

Cells were harvested for RNA isolation at the beginning, middle and end of the expansion process. Total RNA was collected with the use of TriFast Reagent (Peqlab) according to the manufacturer's instructions. Potential genomic DNA contamination was removed by digestion with DNase (Life Technologies) followed by reverse transcription at 50°C for 60 min with the use of Superscript III (Life Technologies) and 250 ng Oligo(dT)$_{18}$-primer (Life Technologies).

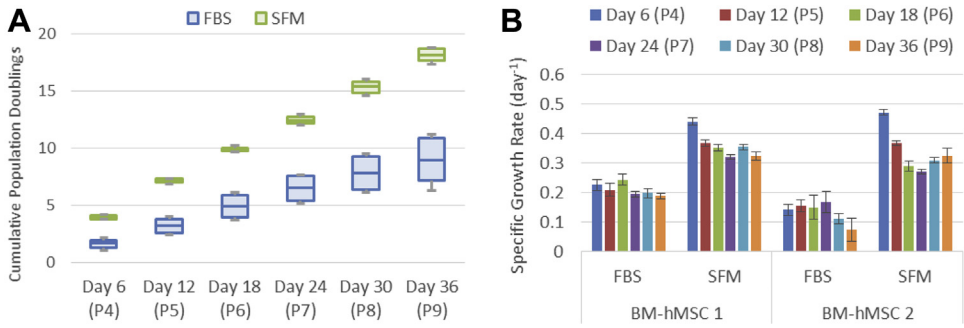

Figure 1. Growth kinetics of the two BM-hMSC lines over 36 days of expansion. (A) Box-and-whisker plots demonstrate the increased consistency and cumulative population doublings in SFM- compared with FBS-containing medium ($P < 0.001$); (B) specific growth rate of hMSCs in FBS and SFM. Data are shown as mean ± standard deviation ($n = 4$).

Quantitative real-time polymerase chain reaction analysis

Quantitative real-time polymerase chain reaction (PCR) was performed with the use of SYBR GreenER quantitative PCR Supermix Universal (Life Technologies); 1× SybrGreen I (Life Technologies) and 0.2 µmol/L primer each on the DNA engine Opticon2 (Biorad) were additionally added, through the use of 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)/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 vascular endothelial growth factor *(VEGF)* (associated with vascularization and growth of blood vessels, NM_001171623.1) (Fw) 5′-GGAAGGAGCCTCCCTCAGGGTTTC G-3′, (Re) 5¢-GCCGGAGTCTCGCCCTCCG G -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).

Human MSC characterization

Immunophenotype analysis was performed by means of multi-parameter flow cytometry before and after the hMSC expansion process with the use of a previously developed protocol [15]. Short-tandem repeat analysis was completed by means of LGC Standards (UK) under their cell line authentication program. Morphology images were obtained with the use of a light microscope (Nikon Eclipse TS-100).

The hMSC differentiation was induced through the use of PRIME-XV differentiation SFM (Irvine Scientific) according to the manufacturer's instructions. After 21 days, the differentiation media were removed and cells were rinsed with PBS fixed with 4% (vol/vol) paraformaldehyde (PFA) at room temperature. Adipocytes were stained with 1% (wt/vol) oil red O (Sigma-Aldrich) in isopropanol at room temperature and rinsed with distilled water. Osteoblasts were incubated with 2.5% (vol/vol) silver nitrate (Sigma-Aldrich) under ultraviolet light (30-min exposure), rinsed with distilled water and stained with fast violet solution (Sigma-Aldrich) containing 4% (vol/vol) napthol AS-MX phosphate alkaline (Sigma-Aldrich) for 45 min at room temperature in the dark. Chondrocytes were stained with 1% (wt/vol) alcian blue (Sigma-Aldrich) in 0.1 mol/L hydrochloric acid (Sigma-Aldrich). After 30-min incubation, cells were rinsed 3 times with 0.1 mol/L HCl. After staining, differentiated cells were visualized under a light microscope (Nikon Eclipse TS-100).

Statistical analysis

Results were deemed to be significant at a value of $P < 0.05$ with the use of a two-tailed Student's *t*-test.

Results

Human MSC growth across multiple donor lines

Human MSCs are currently under clinical investigation for the treatment of many diseases, with the majority of these off-the-shelf therapies typically requiring more than one billion cells per patient

[16,17]. For manufacturing processes to meet this demand, a large cell expansion ratio will be required to treat many patients from the same batch. Figure 1 shows the relative difference between a SFM, PRIME-XV, and media supplemented with FBS in terms of hMSC growth. It is clear that the SFM offers a significantly higher proliferation rate over FBS ($P < 0.001$), with a maximum specific growth rate of 0.471 ± 0.009 per day compared with 0.244 ± 0.018 per day during the 36-day expansion process. This increased growth rate corresponds to a final median cumulative population doubling level of 18.15 in the SFM compared with 8.93 in FBS culture across the two donors at the end of the 36-day expansion process. This represents an increase of approximately 600 times the number of cells under the SFM culture compared with FBS culture over this expansion period, which dramatically increases the effective product yield and potential scalability of the SFM process to meet the needs of a large-scale, off-the-shelf, cell-based therapy. Cells that have nutrient deprivation typically spend longer in the G1 phase of the cell cycle, resulting in slower proliferation [18]. The fact that the PRIME-XV medium supported a more rapid cell growth therefore indicates that it may provide a better nutritional balance or activates growth regulation pathways, such as the PI(3)K pathway, than the FBS-containing medium under the medium exchange regimen used here.

One of the key driving factors for the overall production cost of an hMSC therapy, as well as medium cost, probably will be in the level of donor-to-donor variability in play during the manufacturing process, owing to increased process time and risk of batch failure. The donor lines selected for this study had previously demonstrated large differences in cellular characteristics [4], which will particularly affect the successful development of patient-specific hMSC therapies. Figure 1A shows the inconsistency between the growth characteristics of these hMSC lines, with a range of 4.86 population doublings after the 36-day FBS expansion process, demonstrating a diverging process that occurs in FBS culture. In contrast to this variance experienced in FBS culture and despite a higher number of cumulative population doublings, the SFM culture process had a corresponding range of only 1.45 population doublings between these donors and batch runs after 36 days in culture.

For the development of a patient-specific, cell-based therapy, in which the inter-donor variability must be accounted for during manufacture, this reduction in product variance has a significant benefit to the process. As discussed previously [4], reducing the divergence in donor cell characteristics will alleviate potential bottlenecks in the isolation, expansion and delivery process, an important consideration for cell-based therapy process development. In conjunction with this, a more consistent expansion process will reduce the risk of product batch failure, which for a patient-specific therapy will mean that the patient will not be treated. Table I shows the variability between the two BM-hMSC lines for a hypothetical process requiring a batch size of 350 million cells from a starting population of two million cells [1]. This demonstrates the increased consistency that can be achieved in the SFM process between production batches and between donors, with a reduction in range from 19.4 days in the FBS process to 0.9 days in the serum-free process. This also has advantages for off-the-shelf therapies because it is possible to select material for the expansion process by pre-screening hMSCs and discarding those that do not display sufficient growth kinetics. Under the SFM condition, both of the BM-hMSC lines displayed similar process times between donors and a reduced inter-donor range. This has the potential to lead to reduced costs in the process development phase because fewer donor lines will be excluded (and therefore require testing) compared with FBS culture and will be available for the production process. The reduced process time to achieve this batch size in the serum-free condition will also be advantageous for reducing the overall medium costs because product batches can be manufactured in reduced time. Furthermore, an increase in achievable cell number per batch will also increase the material available for quality release testing, which is an important consideration of patient-specific therapies because each patient batch must be independently tested before release. A study by Deskins *et al.* [19] also demonstrated that three independent *in vitro* assays that were based on growth rate, proliferative potential and ATP content were able to predict *in vivo* performance, with hMSCs performing above average in all three assays having increased *in vivo* regenerative abilities.

Associated with the reduction in inter-donor variability, further reductions in process input

Table I. Process time required to manufacture a theoretical batch of 350M hMSCs, demonstrating variation in process time between and within donor material that is reduced in the serum-free process.

		Per dose of 350M hMSCs		
Condition	hMSC line	Process time (days)	Inter-batch range (days)	Inter-donor range (days)
FBS	BM-hMSC 1	23.5	0.9	19.4
	BM-hMSC 2	39.5	5.0	
SFM	BM-hMSC 1	12.9	0.5	0.9
	BM-hMSC 2	12.4	0.6	

Assumptions: Starting population of 2M hMSCs expanded in the T-flask process presented in this study.

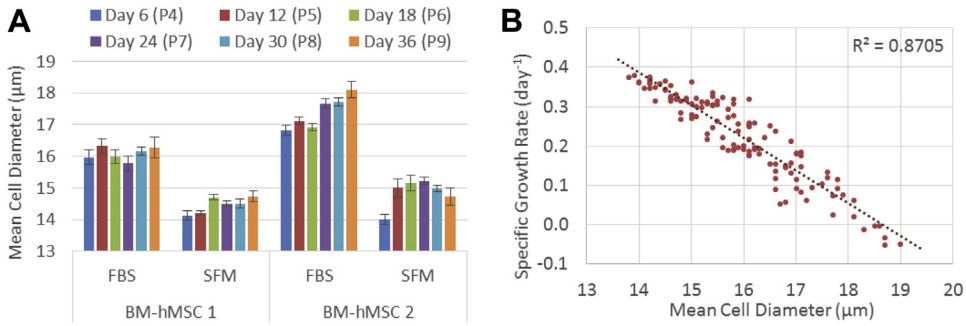

Figure 2. Mean cell diameter of the two BM-hMSC lines over 36 days of expansion. (A) Reduced mean diameter of hMSCs cultured in SFM- compared with FBS-based expansion; (B) correlation showing the relationship between mean cell diameter and specific growth rate of the subsequent passage. Data are shown as mean ± standard deviation ($n = 4$).

variability can be made by placing controls on the supply of raw materials. Considering the potential issues surrounding the limited availability and batch-to-batch variability of FBS [11], the development of defined, SFM formulations will further drive consistency into the manufacturing process. In this model, culture medium can be manufactured to a specific formulation, ensuring inter-batch consistency and reducing overall costs by scaling up the medium manufacturing process and benefitting from increasing economies of scale. This is in contrast to a manufacturing process that is based on FBS, in which the cost of the culture medium will increase as the process is scaled through clinical development into commercial scale production. It should be noted, however, that the development of a defined medium formulation should be based on a rigorous product understanding so that desired product attributes are maximized and not affected by the various medium components. These desired product attributes could range from growth kinetics all the way to functional attributes, which will depend on the target clinical indication.

Human MSC size during the expansion process

The cell diameter of the hMSCs throughout the expansion process in both FBS and the SFM culture has been measured to determine whether this attribute remains consistent. It can be seen from Figure 2 that the hMSCs cultured in the SFM have a smaller diameter throughout the expansion process compared with FBS, which in volumetric terms equates to around half the size (assuming complete sphericity). The increase in cell diameter of BM-hMSCs toward the end of the culture process is associated with a reduction in growth rate (Figure 1) and therefore stability in cell diameter throughout culture will be important to maintain hMSC growth kinetics. Figure 2B shows the relationship between the mean cell diameter of the hMSC population against the growth kinetics of the subsequent passage. This demonstrates that the smaller hMSCs generally have higher growth kinetics, which has been reported previously [20,21]. In addition to growth, hMSC size is also linked to aging of hMSCs and loss in differentiation potential [22,23] and therefore increasing size can also be used as a surrogate marker of hMSC senescence.

Importantly, the osmolality of the FBS-based and PRIME-XV SFM is similar (0.31 and 0.29 Osmol/kg, respectively), further indicating that the difference in cell size noted in Fiure 2A is not a simple matter of a change in osmotic balance, although it should be noted that the osmolality of the PRIME-XV SFM is closer to human physiological conditions [24]. Although the reasons for this relationship are not clear, it can nonetheless provide a basis for evaluating the stability of the hMSCs throughout expansion and provide as an early indication that cell growth kinetics may begin to reduce. The smaller cell size in the SFM will also allow for higher number of cells per area, an important attribute for adherent cell expansion; however, the implications of this smaller size on the functional properties must be assessed during product pre-clinical and subsequent clinical development.

The size of the hMSC also has the potential to affect the post-transplant safety profile of the therapy, and the impact of the process conditions on this attribute should be considered in relation to the delivery method [25]. If the cell-based therapy is to be delivered by intra-arterial infusion, there is a potential risk of micro-embolisms and decreased cerebral blood flow, which must be mitigated by altering the cell dose, infusion volume and velocity [26]. The size of freshly isolated stem cells is ~11 µm and can be transported through capillaries, whereas expansion has previously been shown to increase the size to ~20 µm, obstructing micro-vascular vessels after infusion [27]. Considering that hMSCs cultured under FBS reached a maximum cell diameter of 18.4

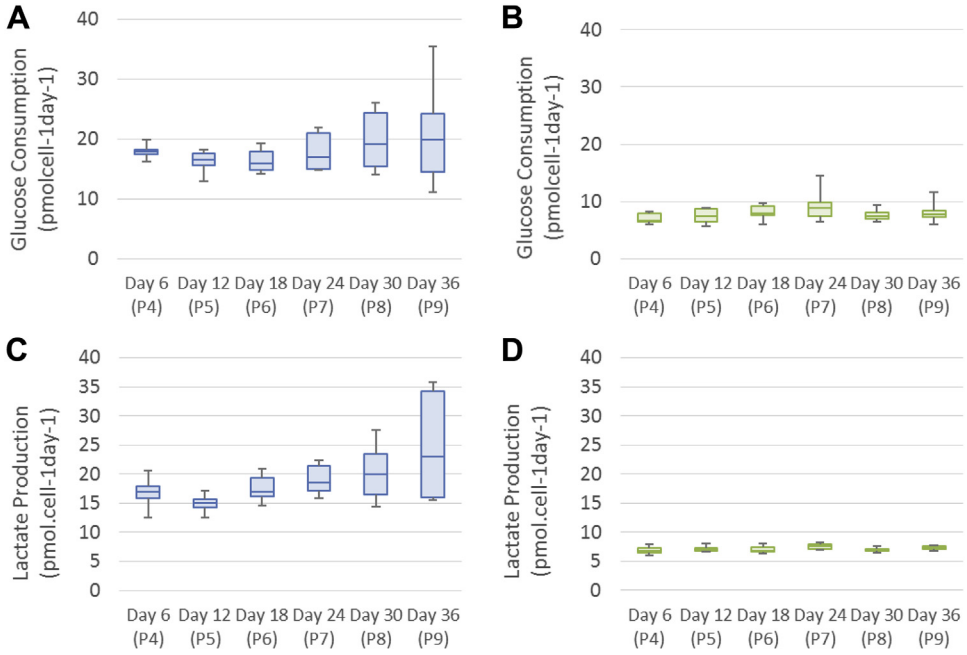

Figure 3. Box-and-whisker plots show per-cell metabolite flux of two BM-hMSC lines over 36 days of expansion. Shown are increased variation of glucose consumption rate in FBS-containing culture (A) compared with serum-free culture (B) and increased variation of lactate production rate in FBS-containing culture (C) compared with serum-free culture (D). Data are shown as $n = 4$ for each BM-hMSC line.

μm compared with SFM with a maximum cell diameter of 15.5 μm, the smaller cell diameter achieved under SFM would offer advantages of reduced risk of these complications. Cells of approximately 15 μm have previously been shown to carry reduced risk of restricting cerebral blood flow, with larger cells requiring a reduced dose and infusion rate [28]. At the end of the expansion process, hMSCs cultured in SFM showed a mean cell diameter of 14.73 ± 0.6 μm (mean ± range) compared with FBS culture, with a cell diameter of 17.19 ± 2.5 μm (mean ± range). The variability in cell diameter throughout expansion in FBS-based medium could have further implications for the delivery process in terms of cell concentration and infusion rate, which probably would have to be standardized during clinical development. As well as an increase in hMSC productivity, this demonstrates a further advantage of SFM culture of hMSCs in terms of cell size consistency, with a smaller cell diameter also maintained throughout our scalable micro-carrier expansion process [13].

Human MSC net metabolite flux

The net metabolite flux of glucose and lactate has been measured in FBS and SFM culture over the expansion process to better understand the relative consistency between the donor hMSCs with time in culture. Figure 3 shows the per-cell flux of glucose and lactate for FBS and SFM culture, with a range of per-cell glucose flux of 24.29 and 8.81 pmol/cell per day for FBS and SFM expansion, respectively. This is concurrent with the relative flux of lactate per cell, with a range of 22.47 and 2.11 pmol/cell per day for FBS and SFM expansion, respectively. This increased range in per-cell metabolite flux in FBS is primarily due to increased variability at the end of the expansion process and is associated with a reduction in hMSC growth and increase in cellular senescence that is not observed in the SFM, despite the higher number of population doublings.

The net flux of metabolites has the potential to form part of a panel of measurements for the purposes of demonstrating comparability, whereby process changes can be evaluated for their impact on cellular characteristics. This understanding of the cell during the expansion process will also be a valuable tool during process scale-up to ensure that the interaction between the cell and its environment has not changed during technology transfer. This highlights the importance of process analytical technology, which can be used to monitor and control process and product attributes to ensure consistency and quality in the final product. It can be seen in Figure 3 that there is reduced per-cell consumption of glucose and production of lactate in SFM culture, which is likely to be a beneficial product attribute as the process is scaled. This will be particularly apparent once suspension-based expansion processes

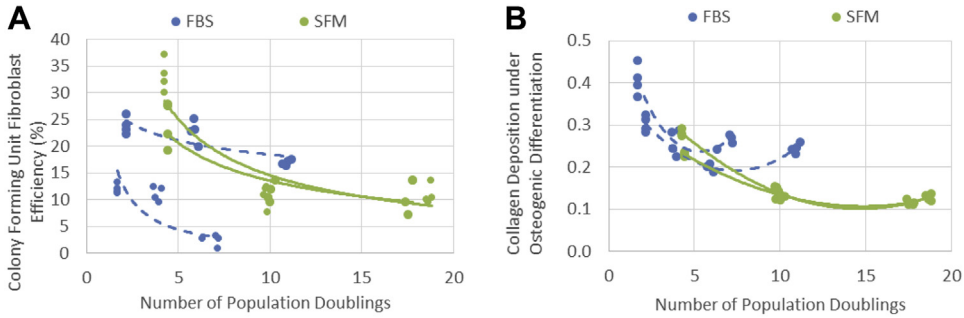

Figure 4. Colony-forming efficiency and osteogenic potential of two BM-hMSC lines against number of population doublings throughout the expansion process. (A) Colony-forming efficiency demonstrates increased consistency between BM-hMSC lines in SFM- compared with FBS-based medium; (B) level of collagen deposition demonstrates maintenance in osteogenic potential of both BM-hMSC lines in SFM at a high number of population doublings.

routinely reach high cell densities (in excess of 1×10^6 cell/mL), in which the buildup of waste products such as lactate has the potential to inhibit the expansion process. Considering that the culture medium is likely to contribute to a significant portion of the cost of goods for hMSC production, product attributes that reduce the usage of culture medium will be increasingly beneficial at the large scale.

Discussion

The increased consistency between donor hMSC lines in terms of per-cell metabolite flux is a further demonstration that the cells cultured in the SFM conditions show reduced inter-donor variation, an important consideration, given the large amount of variation in cell-based therapy manufacture. Although this has been demonstrated in the present study for basic metabolites, there is scope to extend this analysis to a larger panel of metabolic intermediates to provide a detailed understanding of the impact of the process on the hMSC metabolic characteristics during scale-up. The reason for this increased consistency in the SFM has yet to be explored in the literature; however, the combination of hMSCs cultured on fibronectin with serum-free growth medium has been previously shown to activate the platelet-derived growth factor receptor, which is essential for cell migration [29]. Activation of hMSCs in this way provides a potential mechanism for the cell characteristics to converge because the hMSCs are actively forced to utilize specific cellular pathways, as opposed to serum-based culture, in which an abundance of various proteins are available to the cells. This combination of a fibronectin coating with growth medium supplemented with platelet-derived growth factor has been used previously to positively select for smaller, highly proliferative cell populations from bone marrow, termed multipotent adult progenitor cells [30]. This positive selection process could also be contributing to the increase in consistency measured in several different characteristics under serum-free conditions, as discussed further below.

Ensuring consistency in hMSC characteristics

There is currently much discussion about the true identity and desired characteristics of hMSCs for clinical applications and how they elicit their therapeutic mechanism of action [31]. Despite this, some hints come from a graft-versus-host disease study showing that just a few passages can make a significant difference. Human MSCs from passages 1–2 compared with passages 3–4 showed a decrease in patient survival and response, whereas no *in vitro* differences were found [32]. Despite this uncertainty in the application of hMSCs, there is still a need to rigorously characterize the cellular product during the development of an expansion process to ensure the process itself is not having a detrimental impact on the product characteristics while yield is increased. It is important that assay development takes place in parallel with clinical development so that the prediction of clinical effect for a specific target indication can be correlated to process measurements *in vitro*.

As hMSC expansion processes move through the development phase, there is an increasing need to assess the characteristics of the product in relation to the number of population doublings the cells have undergone and is favorable under the current regulatory guidelines. This has the benefit of normalizing cell expansion data because passage number does not take into account the seeding density of the cells or the relative expansion level of the product in each condition. The use of population-doubling level versus cellular characteristic in this way allows for a fair comparison between conditions and is far more amenable to comparisons with scale-up technology

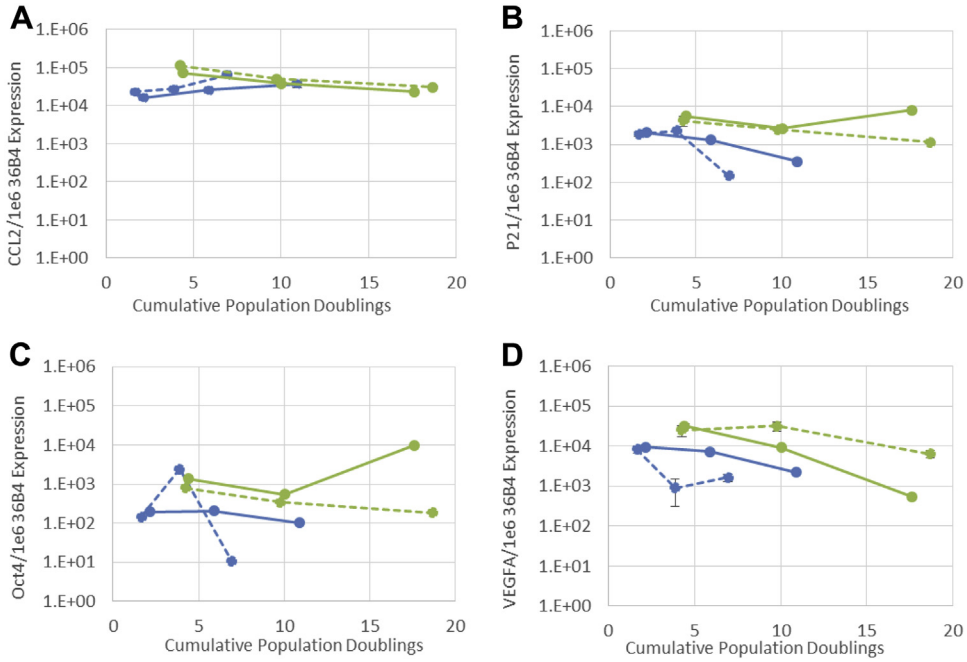

Figure 5. Quantitative real-time PCR analysis shows RNA expression of four hMSC genes for the two BM-hMSC lines in FBS (blue) and SFM (green) expansion. Shown are maintained expression of CCL2 (A), P21 (B) and Oct4 (C) and a reduction in expression of *VEGF*-A (D) in SFM culture. Complementary DNA is normalized to housekeeping gene *36B4*. Solid line indicates BM-hMSC 1; dashed line, BM-hMSC 2.

such as bioreactors, when the term "passage" does not readily apply. Figure 4 shows the colony-forming potential and osteogenic potential of the hMSC donor lines under FBS and SFM culture against the number of population doublings. This demonstrates that both product attributes decrease through the expansion process as the number of population doublings increases, which hints at the challenges that exist when developing large-scale manufacturing processes, which are likely to require lot sizes in excess of a trillion cells [33].

Despite this, hMSCs cultured in SFM retained a similar level of colony-forming potential at a high number of population doublings, and, importantly, the consistency between the two hMSC donor lines was far greater than in FBS. Our data therefore suggest that the SFM condition used, PRIME-XV, in conjunction with growth on fibronectin, is able to support the generation of a more homogenous cell population in terms of colony-forming potential as well as cell size and growth rate, possibly through a positive selection process or the maintenance of asymmetric division. Indeed, an increased presence of CFU-F in an MSC population has previously been noted when an optimized defined medium formulation is used as compared with DMEM/FBS [34]. Furthermore, Wagner *et al.* [35], who compared two serum-containing media, also noted that one was able to support a more homogenous morphology than the other. This maintenance of consistency between donors will be important for both patient-specific and off-the-shelf therapies and will reduce the likelihood of product batch failure during quality testing. This will result in a reduced cost at the large scale because the capital invested per batch will be high, but, more importantly, for patient-specific therapies, a batch failure would result in a patient going without treatment, which would be highly undesirable.

It is also important that the hMSCs retain the expression of key genes throughout the expansion process because they are likely to play a key role in the product performing its function *in vivo*. Figure 5 shows the RNA expression of the donor hMSC lines in FBS and the SFM culture against population-doubling level. It is clear that despite the increased population-doubling level, hMSCs cultured under SFM conditions retained the expression of all four genes analyzed, indicating that the positive selection we believe is occurring to generate the more homogenous population is not affecting the expression of key genes. CCL2 has been implemented in the recruitment of T-cells, monocytes and dendritic cells to the sites of inflammation [36] and is therefore an important gene to maintain for clinical indications that require a level of immune modulation. Figure 5B shows that the relative expression of *P21*, a gene relating to cellular aging and senescence, has not unduly increased throughout the expansion process in SFM or FBS culture. Oct4 is a marker of

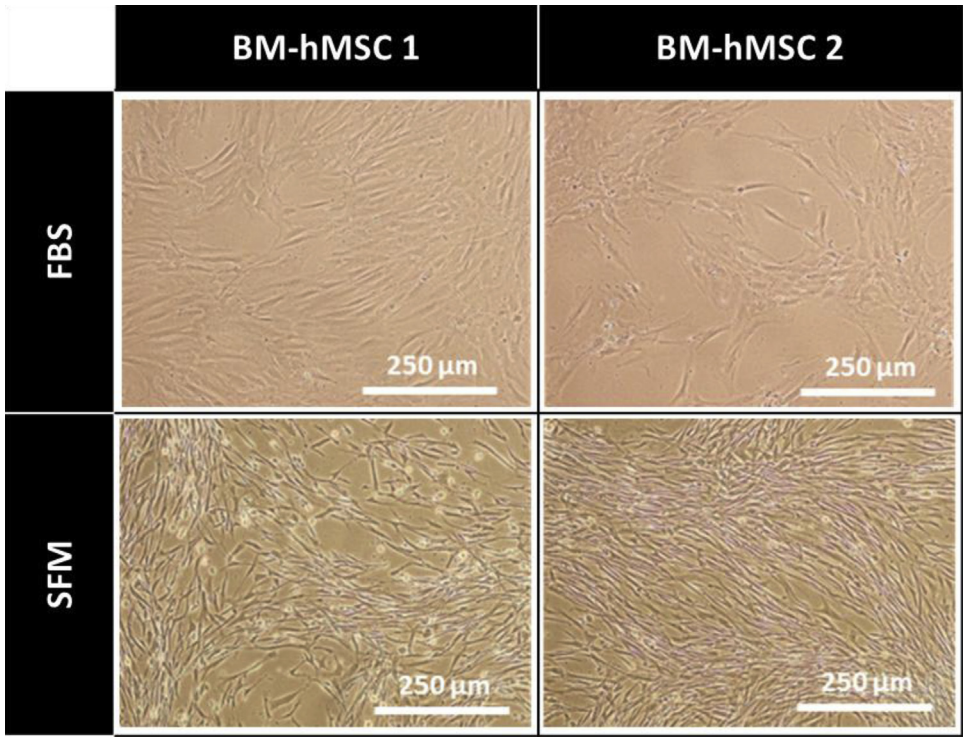

Figure 6. Phase-contrast images of the two BM-hMSC lines show the increased consistency in morphology between the cell lines under SFM expansion compared with FBS.

pluripotency, mainly associated with embryonic stem cells, but has previously demonstrated expression in hMSCs [37]. Maintenance of Oct4 under SFM expansion demonstrates the continued ability of the cells to self-proliferate at high population-doubling levels and will be important for the clinical application of cell-based products that undergo cellular differentiation. *VEGF* has been shown to be a highly important gene in the promotion of angiogenesis by hMSCs [38], which will be important in a number of clinical indications, particularly for cardiac repair [39], a key target for a number of hMSC-based therapies. Despite a higher relative expression of *VEGF* in hMSCs cultured in SFM, there is a decrease in the expression of *VEGF* as the number of population doublings increases, which should be further investigated if the hMSC product requires a high level of cumulative population doublings and is to be used for clinical indications requiring angiogenesis.

Figure 6 shows the difference in hMSC morphology between donor lines in FBS-based culture that is reduced in SFM culture, with smaller spindle-shaped cells. This increased consistency between donors in cellular morphology observed in SFM has benefits for the development of manufacturing processes that are based on a fixed surface area. This will be particularly apparent for patient-specific therapies, in which the number of obtainable cells per square centimeter will determine the final cell yield of the product batch. Considering that manufacturing processes for these cell-based therapies probably will have a minimum number of cells per dose, this reduced variation under SFM will greatly reduce the risk of product batch failure, increasing the cost-efficiency of the process. As well as morphology, the hMSCs have demonstrated the desired immunophenotype and tri-lineage differentiation potential in FBS and SFM culture throughout the entire expansion process (Supplementary Figure 1). Additionally, hMSCs in both conditions have demonstrated the correct genotype profile at the end of the expansion process, as determined by means of short-tandem repeat analysis for each donor cell line (Supplementary Figure 2).

Conclusions

The development of consistent manufacturing processes remains a key challenge that must be overcome to ensure the successful translation of cell-based therapies. SFM has the potential to reduce the variability of input material to these processes, which will allow for increased control over process consistency. By developing a serum-free process, we have increased the yield and consistency of hMSC expansion between donors, which offers large

advantages in the development of both off-the-shelf and patient-specific cell-based therapies. The convergence of hMSC characteristics throughout an expansion process demonstrates a level of control over the product manufacture, which has the potential to increase the cost-effectiveness and reduce the risk in these processes.

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Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.jcyt.2015.08.002.