

Characterization of human mesenchymal stem cells from multiple donors and the implications for large scale bioprocess development



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ABSTRACT

Cell-based therapies have the potential to contribute to global healthcare, whereby the use of living cells and tissues can be used as medicinal therapies. Despite this potential, many challenges remain before the full value of this emerging field can be realized. The characterization of input material for cell-based therapy bioprocesses from multiple donors is necessary to identify and understand the potential implications of input variation on process development. In this work, we have characterized bone marrow derived human mesenchymal stem cells (BM-hMSCs) from multiple donors and discussed the implications of the measurable input variation on the development of autologous and allogeneic cell-based therapy manufacturing processes. The range of cumulative population doublings across the five BM-hMSC lines over 30 days of culture was 5.93, with an 18.2% range in colony forming efficiency at the end of the culture process and a 55.1% difference in the production of interleukin-6 between these cell lines. It has been demonstrated that this variation results in a range in the process time between these donor hMSC lines for a hypothetical product of over 13 days, creating potential batch timing issues when manufacturing products from multiple patients. All BM-hMSC donor lines demonstrated conformity to the ISCT criteria but showed a difference in cell morphology. Metabolite analysis showed that hMSCs from the different donors have a range in glucose consumption of 26.98 pmol cell⁻¹ day⁻¹, Lactate production of 29.45 pmol cell⁻¹ day⁻¹ and ammonium production of 1.35 pmol cell⁻¹ day⁻¹, demonstrating the extent of donor variability throughout the expansion process. Measuring informative product attributes during process development will facilitate progress towards consistent manufacturing processes, a critical step in the translation cell-based therapies.

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1. Introduction

Regenerative medicine is an expanding field, which includes the use of living cells and tissues as medicinal therapies to treat currently unmet clinical need. Cell-based therapies, a platform technology, form a significant part of this field with many treat-

Abbreviations: BM-hMSC, bone marrow derived human mesenchymal stem cells; MNC, mononuclear cells; FBS, fetal bovine serum; DMSO, dimethylsulphoxide; CFU-f, colony forming unit fibroblast; QC, quality control; QA, quality assurance; STR, short tandem repeat; CQA, critical-to-quality attributes; IL-6, interleukin 6; IDO, indoleamine 2,3-dioxygenase; MoA, mechanism of action; PAT, process analytical technology.

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ments for diseases such as cancer, diabetes and stroke currently progressing through clinical development [1]. Human mesenchymal stem cells (hMSCs) are a leading candidate for many of these clinical indications due to their relative ease of isolation, potential for *ex vivo* expansion and their ability to secrete a range of trophic factors which can initiate the regeneration of many tissues. For the majority of these clinical indications, however, the *in vitro* expansion of cells is required in order to deliver an effective therapeutic dose [2]. The intention of this expansion step is to manufacture a sufficient number of cells to deliver therapeutic benefit without having a detrimental impact on the quality of the cell. Cell-based therapies therefore represent a step change from current pharmaceutical production, where the product of interest is usually a protein, not the cell itself. This introduces a range of additional challenges for the field, not least of all how a cell-therapy bioprocess can

be developed to consistently manufacture products from multiple donors.

Autologous cell-based therapies, where the cell donor and recipient is the same individual, are patient specific and their manufacture must be scaled-out to ensure that patient material is segregated and cross-contamination of material is avoided. Scale-out of autologous therapies will likely necessitate multiple manufacturing facilities, creating the need for local automation and the demonstration of comparability between these sites [3,4]. The main advantage of autologous cell-based therapies is the lack of immune rejection associated with donor transplant material, eliminating the need for immunosuppressive medication, which would add significant cost to the treatment. Autologous therapies may also benefit from the development of point-of-care devices, where functional closed devices can be used to manufacture cell-based therapies at the bedside. These typically involve the isolation and enrichment of cells directly from the patient and are returned on-site as “minimally manipulated” therapies. Despite these advantages, many challenges remain in the development and commercialisation of autologous cell-based therapies. It is possible that the route of the target disease might be with the patient’s own cells and it would therefore be better to avoid using them, or indeed the patient is unable to undergo the procedure required for cell isolation. Furthermore, issues surrounding the quality test burden and logistics add to the complexity for the production and delivery of a cost effective autologous cell-based therapy and failure of product batches would be likely to lead to an inability to treat patients.

Regenerative cell-based therapies where the donor and the recipient are different individuals are termed “allogeneic”. This creates an off-the-shelf business model, which is far more akin to current biopharmaceuticals, representing an attractive commercial opportunity. Assuming cell products can be stored long-term *i.e.* their manufacture is decoupled from delivery to the patient; the cells can be made available on demand. In contrast to autologous therapies, allogeneic products have the potential to be scaled-up, potentially benefitting from the economies of scale experienced by traditional bioprocesses [5,6]. As such, manufacturing technologies employed for allogeneic therapies are likely to differ in terms of nature and scale including the use of traditional scale-up technology such as stirred-tank bioreactors [7,8]. Allogeneic therapies will however create a product that is “more than minimally manipulated”, which means that the regulatory pathway requires far more time and resource to complete.

For the development of both autologous and allogeneic cell-based therapies, the characterization of the input material from different donors will be necessary to assess any potential variation in the manufacturing and delivery process. The aim of this study, therefore, is to characterize the input material for an hMSC therapy bioprocess and then assess the implications for the development and operation of a large scale “more than minimally manipulated” cell-based therapy bioprocess.

2. Materials and methods

2.1. Culture of hMSCs

Bone marrow derived human mesenchymal stem cells (BM-hMSCs) were purchased or donated as mononuclear cells (MNCs) from Lonza (USA) after the patient had given informed consent. The local Ethical Committee approved the use of the sample for research. MNCs from Lonza were initially plated at a cell density of 100,000 MNCs cm^{-2} with the first medium exchange taking place on day 2 and every 3 days thereafter. Human MSCs were cryopreserved at passage 0 to passage 2 at a density of either 1×10^6 cells ml^{-1} or 2×10^6 cells ml^{-1} in a freeze

medium containing 90% (v/v) FBS (Hyclone, Belgium) and 10% (v/v) dimethylsulphoxide (DMSO). Cells suspended in 1 ml freeze medium were placed into 2 ml cryovials and placed in a -80°C freezer to passively cool at a rate of 1°C min^{-1} . After 24 h, cells were placed in the vapor phase of a monitored liquid nitrogen cryostorage bank for long-term storage.

Cells were grown in T-flasks seeded at 5000 cells cm^{-2} in a humidified atmosphere at 37°C in air containing 5% CO_2 . Cells were cultured in Dulbecco’s Modified Eagle Medium (DMEM; Lonza, UK) supplemented with 10% (v/v) fetal bovine serum (FBS; Hyclone) and 2 mM Ultra-glutamine (Lonza, UK). A complete medium exchange was performed on day 3 and cells were passaged at day 6 of culture (established time to confluency). On passage, the BM-hMSCs were washed with Ca^{2+} and Mg^{2+} free phosphate buffered saline (PBS) (Lonza, UK) and then incubated for 5 min with trypsin (0.25 %)/EDTA solution (Lonza, UK) to aid cell detachment from the culture surface. Trypsin was then inactivated by the addition of fresh growth medium equivalent to $3 \times$ the volume of the trypsin solution used for cell detachment. The cell suspension was then centrifuged at $220 \times g$ for 5 min at room temperature, the supernatant discarded and the remaining pellet re-suspended in an appropriate volume of culture medium. Viable cells were counted and an appropriate number of cells were then re-seeded into a fresh tissue culture flask.

2.2. Analytical techniques

Cell counting and viability (via propidium iodide exclusion) was performed using a NucleoCounter NC-100 automatic mammalian cell counter (Chemometec, Denmark). To assess the metabolic activity of the BM-hMSCs during culture, 1 ml media samples were taken from each T-75 culture flask, stored initially at -18°C and transferred to -80°C for permanent storage. Multiple media samples were thawed, randomised and analysed for glucose (g/L), lactate (g/L) and ammonium (mmol/L) using the BioProfile FLEX (Nova Biomedical, USA).

2.3. Immunophenotype analysis

Immunophenotype analysis was performed by multiparameter flow cytometry before and after hMSC expansion using a previously developed protocol [9]. Briefly, detached cells were suspended in growth medium and loaded onto a 96 well plate. The plate was centrifuged for 5 min at $220 \times g$. The aspirate was removed and the cells re-suspended and washed in flow cytometry staining buffer (R&D Systems, UK) and the centrifugation cycle repeated. The cells were stained for 30 min in the dark at room temperature with fluorescent monoclonal antibodies against CD34 (PE-CY5), CD73 (PE-Cy7), CD90 (APC), CD105 (PE) and HLA-DR (FITC, all from BD Biosciences, UK) in addition with the corresponding isotype controls. All data was obtained using a Guava easyCyte 8HT flow cytometer (Merck Millipore, UK) equipped with 488 and 640 nm excitation running guavaSoft Incyte acquisition software (v2.5). A minimum of 10,000 gated (Forward scatter/Side scatter) events were recorded for each sample. Post-acquisition analysis and compensation was performed with FlowJo v7.6.5 (Treestar Inc, USA) software.

2.4. Tri-lineage differentiation potential

The hMSC differentiation was induced using StemPro Differentiation Medium (Thermo Fisher, UK) as per the manufacturer’s instructions. After 21 days the differentiation media were removed, cells rinsed with PBS then fixed with 4% (v/v) PFA at room temperature. Adipocytes were stained with 1% (w/v) Oil Red O (Sigma–Aldrich, UK) in isopropanol at room temperature and rinsed with distilled water. Osteoblasts were incubated with 2.5%

(v/v) silver nitrate (Sigma–Aldrich, UK) under ultraviolet light (30 min exposure), rinsed with distilled water and stained with fast violet solution (Sigma–Aldrich, UK) containing 4% (v/v) naphthol AS-MX phosphate alkaline (Sigma–Aldrich, UK) for 45 min at room temperature in the dark. Chondrocytes were stained with 1% (w/v) Alcian blue (Sigma–Aldrich, UK) in 0.1 M hydrochloric acid (Sigma–Aldrich, UK). After 30 min incubation, cells were rinsed three times with 0.1 M hydrochloric acid (Sigma–Aldrich, UK). After staining, differentiated cells were visualised under a light microscope (Nikon Eclipse TS-100, UK).

2.5. Quantitative osteogenesis assay

Osteogenesis was quantified by hMSC collagen production using the Sircol Assay (Biocolour, UK) following osteogenic differentiation. Collagen standards of acid-soluble collagen Type I at 0, 0.1, 0.2 and 0.4 g L⁻¹ were used to quantify the collagen production. BM-hMSCs were seeded at 10,000 cells cm⁻² in a 12 well plate with the previously described cell culture medium; after 3 days culture growth medium was exchanged to PRIME-XV[®] Osteogenic Differentiation Serum-Free Medium (SFM) (Irvine Scientific, USA) 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, UK) and 9% formaldehyde (v/v) (Sigma, UK) for 30 min at room temperature. The monolayer was washed and Sircol Dye Reagent (Biocolour, UK) was added to each well for 30 min, removed and the cell monolayer was washed with Acid-Salt Wash Reagent (Biocolour, UK). Alkali Reagent (Biocolour, UK) 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, UK) at an absorbance of 555 nm.

2.6. Colony-forming unit fibroblast (CFU-f) efficiency

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

2.7. IL-6 production assay

Human MSCs were seeded into T-flasks and maintained as described above. On day 3, the medium was removed and the cells were washed once with PBS. Fresh medium was added containing 10 ng/ml of recombinant human interferon- γ (IFN- γ) and tumour necrosis factor- α (TNF- α) (both from Invitrogen, UK). After 24, 48 or 72 h the medium was harvested and analysed via ELISA. IL-6 concentration was measured with human IL-6 ELISA Set (BD Biosciences, UK) according to the manufacturer's instructions. Briefly, capture IL-6 antibody was coated onto Maxisorp 96-well plates. Samples and standards were loaded on the plates and incubated for two hours at room temperature followed by washing with buffer solution. The wells were then incubated with biotinylated anti-human IL-6 antibody and Streptavidin–horseradish peroxidase conjugate (SAV-HRP). Substrate solution containing tetramethylbenzidine (TMB) and hydrogen peroxide was then added to each well for 30 min in the dark. Stop solution was then added to each well. Absorbance was read at 450 nm using a FLUOstar Omega plate reader (BMG Labtech, UK).

2.8. Kynurenine assay

150 μ l of medium used for the IL-6 assay was incubated with 50 μ l of 30% (vol/vol) trichloroacetic acid (Sigma–Aldrich, UK) for 10 min at 50 °C, vortexed and centrifuged at 10,000 \times g for 5 min. 75 μ l of the supernatant was transferred to a 96-well plate followed by addition of equal volume of Ehrlich's reagent (1% w/v *p*-dimethylbenzaldehyde in glacial acetic acid) (Sigma–Aldrich, UK) for 10 min at room temperature. Optical absorbance was measured at 492 nm. The amount of kynurenine was determined using a standard curve from 0 to 100 μ M.

2.9. Short tandem repeat (STR) analysis

Short tandem repeat analysis was completed by LGC Standards (UK) under their cell line authentication program.

$$\text{CFU Efficiency} = \left(\frac{\text{Number of colonies formed}}{\text{Number of cells seeded}} \right) \times 100 \quad (1)$$

$$\text{Specific Growth Rate, } \mu = \frac{\ln(C_x(t)/C_x(0))}{\Delta t} \quad (2)$$

Where μ = specific growth rate (h⁻¹), $C_x(t)$ and $C_x(0)$ = cell numbers at the end and start of exponential growth phase respectively and t = time (h)

$$\text{Population doubling} = \frac{1}{\log(2)} \times \log \left(\frac{C_x(t)}{C_x(0)} \right) \quad (3)$$

Where $C_x(t)$ and $C_x(0)$ = cell numbers at the end and start of exponential growth phase respectively and t = time (h)

$$\text{Fold increase} = \frac{C_x(f)}{C_x(0)} \quad (4)$$

Where $C_x(f)$ = final cell number at the end of passage and $C_x(0)$ = initial cell number

$$\text{Specific metabolite flux, } q_{\text{met}} = \frac{\mu}{C_x(0)} \times \frac{C_{\text{met}}(t) - C_{\text{met}}(0)}{e^{\mu t} - 1} \quad (5)$$

Where q_{met} = specific metabolite flux, μ = specific growth rate (day⁻¹), $C_{\text{met}}(t)$ and $C_{\text{met}}(0)$ = concentration of metabolite at the start and end of exponential growth phase respectively, $C_x(0)$ = cell number at the start of exponential growth phase and t = time (day)

$$\text{Lactate yield from glucose, } Y_{\text{Lac}/\text{Gluc}} = \frac{\Delta \text{Lac}}{\Delta \text{Gluc}} \quad (6)$$

Where $Y_{\text{Lac}/\text{Gluc}}$ = lactate yield from glucose, ΔLac = lactate production over specified time period and ΔGluc = glucose consumption over same time period.

2.11. Statistical analysis

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

3. Results and discussion

3.1. Autologous bioprocess development

As mentioned previously, autologous cell-based therapy products must be scaled-out to meet demand and the process must have the capability to deal with innate variation that exists when manufacturing each product batch from a different donor. With this in mind, the variation in input material should be assessed in order to inform product and process development, increase efficiency and reduce costs [10]. In line with our previous work aimed at developing a quantitative approach for hMSC culture, we expanded upon this by including multiple hMSC donor cells (Table 1) as well as

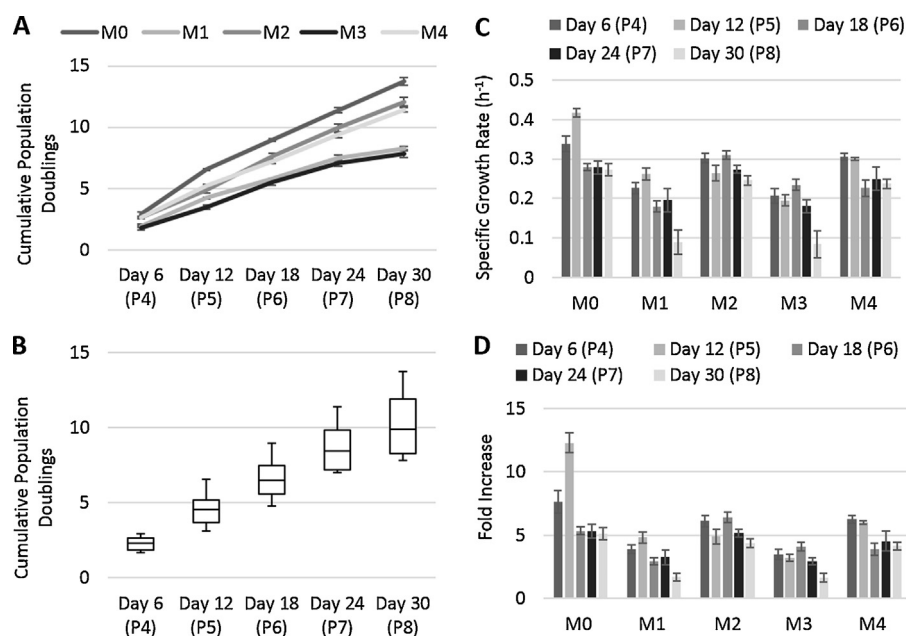


Fig. 1. Growth potential of the five BM-hMSC lines over five passages. Showing (A) Cumulative population doublings; (B) variation in cumulative population doublings across all six BM-hMSC lines; (C) specific growth rate; (D) fold increase. (Data shows mean \pm SD, $n = 4$).

Table 1
Summary of BM-hMSC line nomenclature and donor information.

hMSC line nomenclature	Donor age	Donor gender	Donor ethnicity
M0	20	Male	Black
M1	27	Male	Black
M2	19	Female	Black
M3	24	Male	Caucasian
M4	25	Female	Hispanic

new analytical techniques [11]. The variation in growth kinetics of the five BM-hMSC lines can be seen in Fig. 1A, with 13.74 ± 0.33 cumulative population doublings achieved by M0 over 30 days in culture, compared to M3 which only achieved 7.81 ± 0.32 over the same period of time. The measured variation in growth kinetics will likely have implications for hMSC products that require culture expansion, as a minimum number of cells will be required to be generated within a set time frame, to meet the specification of the product. With such differences in the growth of BM-hMSCs between donors this makes the manufacture of autologous cell-based products a real challenge and solutions have been proposed to alleviate this, such as reducing the expansion of the product and utilising functionally closed automated manufacturing devices [12]. The implications of this difference in growth kinetics will also impact the logistics and timing, as the process will typically have to operate at the “worst-case-scenario”. Table 2 shows the variation in process time for each of these donor hMSC lines for a theoretical process with the batch requirement of 350 million hMSCs [1]. The range in the process time between these donor hMSC lines for this hypothetical product is over 13 days, creating potential batch timing issues when manufacturing products from multiple patients. This also creates differences in the medium utilisation to achieve each patient dose, for example M1 requires almost double the volume of medium per million cells compared to M0 (Table 2). This will likely impact of the overall cost of goods for patient specific processes, as medium and particularly the serum component of medium is likely to be a key cost driver during process scale-out [13]. Table 2 also shows the inter batch range for each of these

Table 2
Process time required to manufacture a theoretical batch of 350 M hMSCs demonstrating variation in process time between and within donor material. Assumptions – starting population of 2 million hMSCs, expanded in the T-flask process presented here.

hMSCs line	Per dose of 350M hMSCs			
	Process time days	Number of passages	Inter batch range (days)	ml culture media per million cells
M0	14.2	3	0.5	87
M1	23.3	4	5.0	159
M2	17.6	4	1.6	117
M3	27.4	5	6.8	151
M4	18.7	4	0.9	129

donor hMSC lines, with cells requiring higher processing times generally showing increased variation. Increased inter batch range has the potential to reduce productivity as process timing will need to be flexible enough to accommodate this variation and production rates are likely to be decreased.

The logistics of isolating cells from the patient, processing these cells and returning them to the patient will have to take place in a limited time period, for example, Provenge® (Dendreon Corporation, USA) a non-expanded cell-based therapy has a processing time of up to 18 h. Process timings must therefore be clearly defined which will be challenging when the difference in growth kinetics is so variable and the process must be run assuming the minimum possible expansion rate of the product to avoid creating a production bottleneck. The implications of this are that low growth rate cells greatly reduce the efficiency of the process, which is likely to increase the cost of developing autologous cell-based products.

The relative metabolite production rate and nutrient consumption rate can be seen in Fig. 2, with the per cell metabolite flux showing differences across the five BM-hMSC lines over the 30 days in culture. The net glucose consumption rate for the BM-hMSC lines remained relatively stable throughout the 30 days of culture with the exception of M3, which showed an increase at the end of the culture period. The increase in net glucose consumption of M3 is linked with an increase in the net lactate production rate (Fig. 2b).

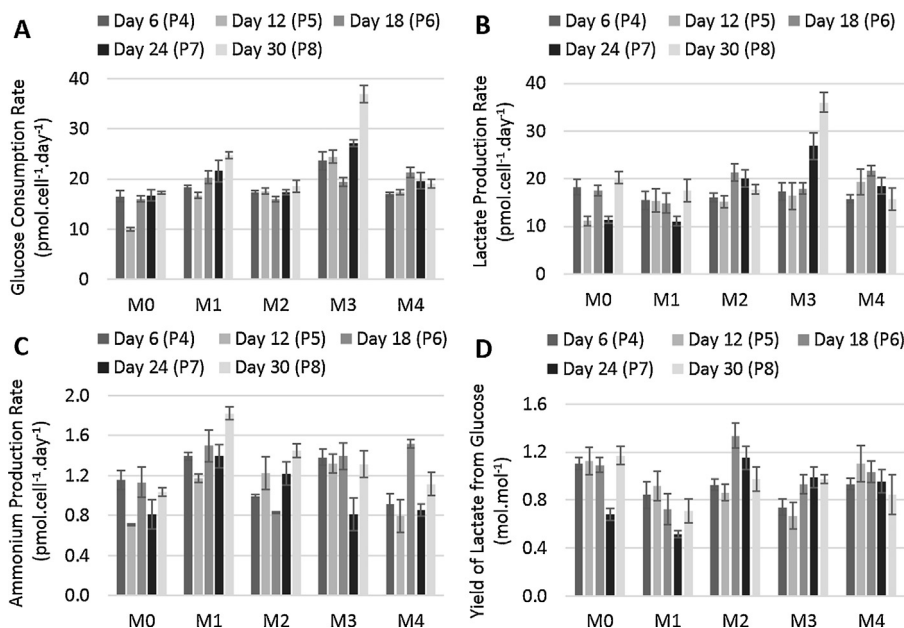


Fig. 2. Metabolite consumption and production of the five BM-hMSC lines over five passages. Showing (A) Specific glucose consumption rates; (B) specific lactate production rates; (C) specific ammonium production rates; (D) specific yield of lactate from glucose. (Data shows mean \pm SD, $n=4$).

This is primarily associated with a reduction in the proliferative rate of the M3 cell line towards the end of the culture process. Variation in net metabolite flux across the cell lines has implications for the operation and control of autologous bioprocesses, as this will change the required nutrient feeding strategy employed during the manufacturing process. Furthermore, an increase in the lactate production of M3 (Fig. 3) has the potential to cause the build-up of toxic components that would need to be limited either by dilution or removal from the process. If the levels of nutrient utilization and metabolite production vary between donors in this way, control systems must be developed and integrated within the manufacturing process with the ability to neutralize this effect and maintain a consistent process, which will be critical for the successful regulation of an autologous manufacturing process.

Morphology has been used for decades as a qualitative assessment of hMSC identity and has also been used as part of the release test for approved cell-based therapies, for example Carticel® (Genzyme, USA) includes morphology as part of their wider product release tests. Fig. 4 shows the difference between the morphology of BM-hMSC lines M2 and M3 after five days in culture, with clear differences between them. The implications for this difference in cell morphology can be appreciated when considering that an adherent cell manufacturing process will be based upon a fixed surface area for cell expansion. With such differences in cell morphology in terms of size and alignment, the effective number of cells per square centimeter of these two cell lines when confluent varies greatly, creating an issue for these manufacturing processes based upon a fixed surface area. In these processes, the final cell number at harvest will vary greatly between patients due to these morphological differences. Considering that manufacturing processes for autologous cell-based therapies will likely have a minimum number of cells per dose, this variation will greatly increase the risk of suffering product batch failure as this minimum number of cells per dose may not be met. Increased batch failure rate will likely increase the inherent risk and inevitably the cost of autologous cell-based therapy products. In addition to the challenges relating to the number of cells obtainable per unit area, the cell size will likely play a role in the downstream processing and delivery of the cells to the patient [8]. The isolation procedure and subsequent positive selection of input material for these patient specific

processes will be critical in reducing the failure rate of these manufacturing processes. Robust and reproducible isolation protocols need to be established to minimize variability of source material and selection criteria based on desired product attributes should be identified at an early stage of development. These isolation and selection processes will likely reduce process variation by placing controls on the input to the process, which will be critical to the success of these personalized cell-based therapies.

As well as the cost implications of a product batch failure it is also important to consider the implications of such an occurrence in terms of not being able to treat a patient with an autologous therapy [14]. This represents a limitation to the use of such therapies and creates a challenge that has not been experienced with traditional medical treatments. Depending on the severity of the clinical indication, this has the potential to cause complications and repetition of the isolation, expansion and delivery process may not be possible. It is clear that autologous therapies represent an opportunity to deliver cell-based therapies from a scale-out process and require innovation beyond current biopharmaceutical manufacture, however, subtle differences in this approach as highlighted above must be considered during product development and commercialization.

3.2. Allogeneic bioprocess development

As described above, the development of allogeneic cell-based therapies represents an off-the-shelf business model more akin to current biopharmaceutical production. These allogeneic processes can be scaled-up to treat multiple patients from a single batch which is likely to increase the cost-effectiveness of the product due to increasing economies of scale, simpler supply logistics and larger amounts of material for quality control (QC)/quality assurance (QA) testing in comparison to autologous processes. Material from multiple donors must therefore be assessed in order to create a master cell bank with enough material to manufacture cells to meet the commercial demand for the cell-based therapy.

Fig. 1b shows the box and whisker plots for the variation across five BM-hMSC lines, which increases with passage number and time in culture. The divergence in cell growth will create an issue for processes with a high expansion ratio, as a consistent process will be harder to obtain the longer the cells are in culture. This high-

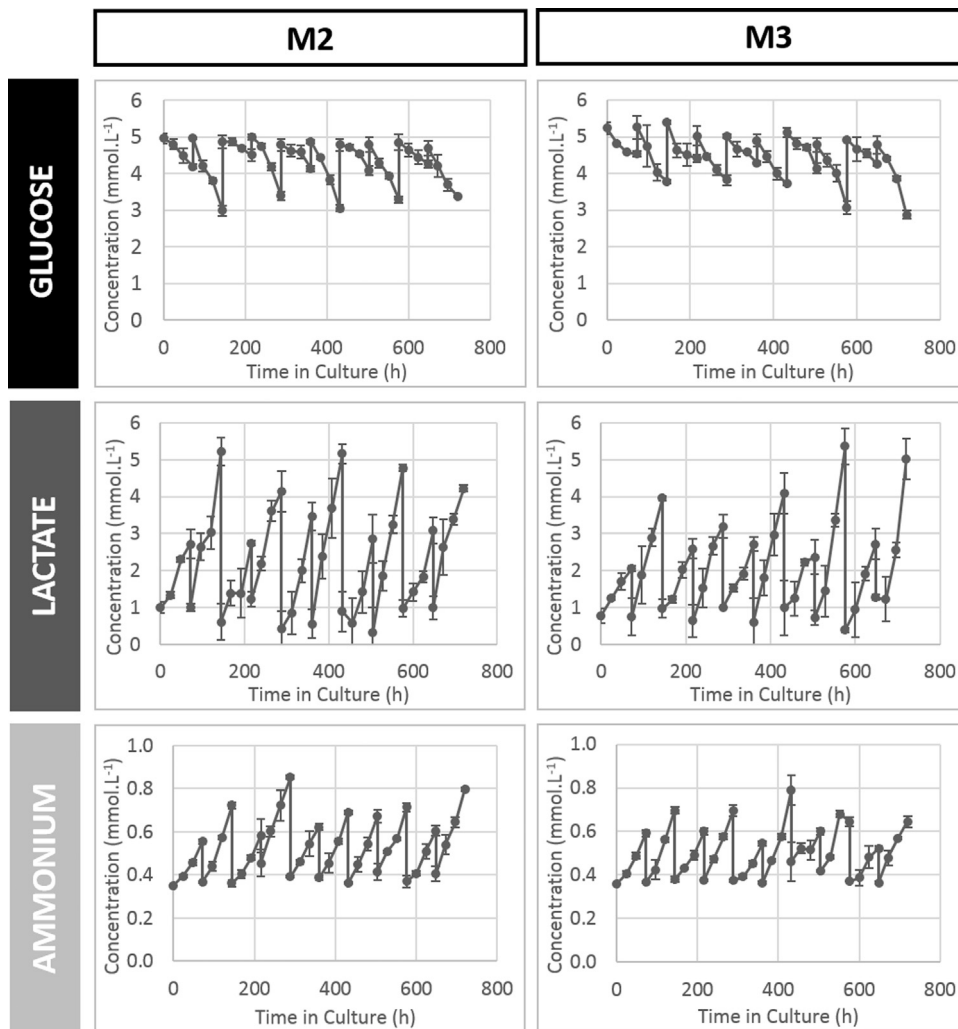


Fig. 3. Metabolite consumption and production showing the difference between the M2 and M3 BM-hMSC lines over five passages. Daily media samples were taken and analysed for glucose, lactate and ammonium concentration. (Data shows mean \pm SD, $n = 4$).

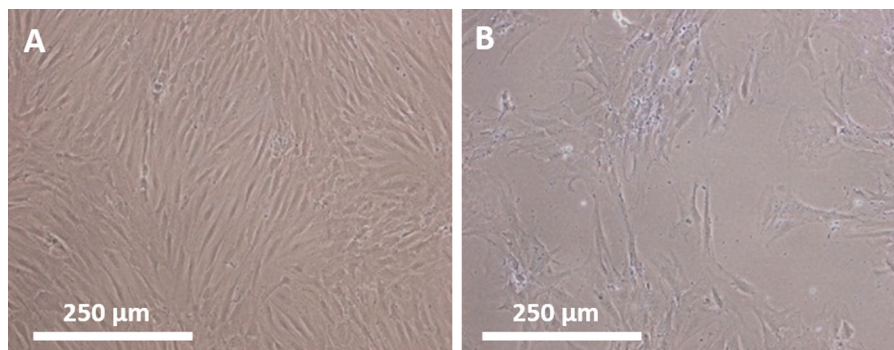


Fig. 4. Phase contrast image showing the difference in BM-hMSC day six morphology between the M2 (A) and M3 (B) cell lines.

lights the importance of developing a process control strategy that is capable of reducing this divergent culture, minimizing the time required for *in vitro* expansion, or ideally producing a convergent process, once it is transferred to a large-scale bioreactor system. As well as process parameters such as dissolved oxygen concentration and pH, this control system must be able to maintain a stable level of nutrients and metabolites, which will be different for each cell line, as identified in Fig. 3. Control of nutrients and metabolites will become particularly important if the bioreactor process is operated at high cell densities, as the consumption of

nutrients and production of metabolites will occur at an increasing rate. The divergence in growth characteristics of BM-hMSCs from multiple donors has implications for the development of allogeneic cell-based therapy manufacturing processes, as they will require a high cell expansion ratio at large scale in order to produce sufficient cell numbers to meet the product demand. Furthermore, as the cells are cultured for a longer period of time, the number of manipulations increases, which has the potential to introduce increased variability into the process. Automated and closed processes have the potential to reduce the inherent variability in each

Table 3

Multiparameter flow cytometry showing percentage co-expression of CD90+, CD73+, CD105+, CD34- & HLA-DR- of each BM-hMSC line at 0 h (passage 1) and after 720 h (passage 5). Mean value \pm SD, in all cases 10,000 events were measured.

hMSC line	Day 0 (P3) culture	Day 30 (Pg) culture
M0	98.97 \pm 0.19%	97.78 \pm 2.26%
M1	96.21 \pm 0.22%	95.97 \pm 0.42%
M2	98.19 \pm 0.09%	95.13 \pm 2.53%
M3	93.60 \pm 2.51%	96.56 \pm 0.42%
M4	98.14 \pm 0.94%	93.17 \pm 1.60%

of these process manipulations and are likely to play a key role in the development of allogeneic cell-based therapies.

The development of an allogeneic product also necessitates a master cell bank, from which the final product can be manufactured [15]. The cost of developing a master cell bank is typically high and cannot be recovered until the product receives market approval, adding to the funding gap in development of allogeneic cell-based therapies. The quality and consistency of the master bank is critical, as once it has been created it cannot be changed and therefore a high level of product and process understanding is required, coupled with rigorous safety and quality testing to ensure that the product in the master cell bank is suitable for the manufacture of the cell-based therapy product.

3.3. Product characterization

Perhaps one of most important aspects of the manufacture of cell-based therapy products is the definition and measurement of cell characteristics [16]. These can be broken down into identity, potency, purity and safety which must all be considered during product development [17]. Despite the differences in cell growth and net metabolite flux described in Sections 3.1 and 3.2, all of the BM-hMSC lines displayed the expected immunophenotype by the positive co-expression of CD73, 90 and 105 and negative co-expression of CD34 and HLA-DR at the start and end of the culture process (Table 3). This is combined with the demonstration of BM-hMSC tri-lineage differentiation potential, an example of this is shown for M2 and M3 cell lines in Fig. 5, demonstrating that the BM-hMSC lines meet the minimum criteria as defined by the International Society of Cellular Therapy (ISCT) [18], despite the differences discussed in Sections 3.1 and 3.2. Short tandem repeat (STR) analysis of these BM-hMSC lines shows that they have retained the 16 key *loci* they are expected to express, indicating that

all of the cell lines have retained the characteristic genotype of BM-hMSCs throughout the entire culture process (see Supplementary material).

It is widely acknowledged that a better understanding of the mechanism by which BM-hMSCs elicit their therapeutic action will be required before processes can be developed in order to preserve or potentially maximize it. Fig. 6a shows the colony forming efficiency of three of the BM-hMSC lines, M2, M3 and M4. Despite similarities in growth kinetics between M2 and M4 their ability to form colonies over ten passages in culture is very different, which would pose a challenge if the products were being assessed under the same QA guidelines. If the product does not meet the QA specification defined during clinical development, the product batch will be failed, creating significant consequences for both autologous and allogeneic processes. As allogeneic manufacturing processes are operated at larger batch sizes, the failure to meet the product release criteria will result in the loss of a large amount of invested capital, increasing the financial risk in the process. In contrast, the implications of a product batch failure during an autologous cell-based therapy process are that the patient will go untreated. Depending on the severity of the clinical indication targeted, this has the potential to be fatal, which will have severe implications for the health of the patient.

The osteogenic potential of M2, M3 and M4 has also been quantified using collagen production under nine days of osteogenic differentiation (Fig. 6b). As with the colony forming unit fibroblast efficiency, the relative production of collagen under osteogenic conditions reduced significantly for M3 and M4 from passage three to passage ten, whilst the collagen production from the M2 cell line remained lower throughout culture. The loss in hMSC multipotency during *in vitro* expansion has previously been related to a loss of *in vivo* bone formation [19], which could be used as a critical-to-quality attribute (CQA) for a clinical indication relating to bone tissue regeneration. Considering the rapid loss in activity after passage three, this would potentially limit the use of culture expanded BM-hMSCs for this type of clinical indication.

Aside from these more traditional clinical indications for BM-hMSCs based on differentiation, there has been growing clinical evidence to suggest that part of their putative mechanism of action lies within their ability for immune modulation, suppressing tissue rejection by inhibiting the response of the patient's lymphatic cells [20,21]. The response of the M2, M3 and M4 cell lines to a pro-inflammatory environment has been assessed by the production of interleukin 6 (IL-6) and kynurenine throughout the culture process. IL-6 has been previously reported to inhibit differentiation

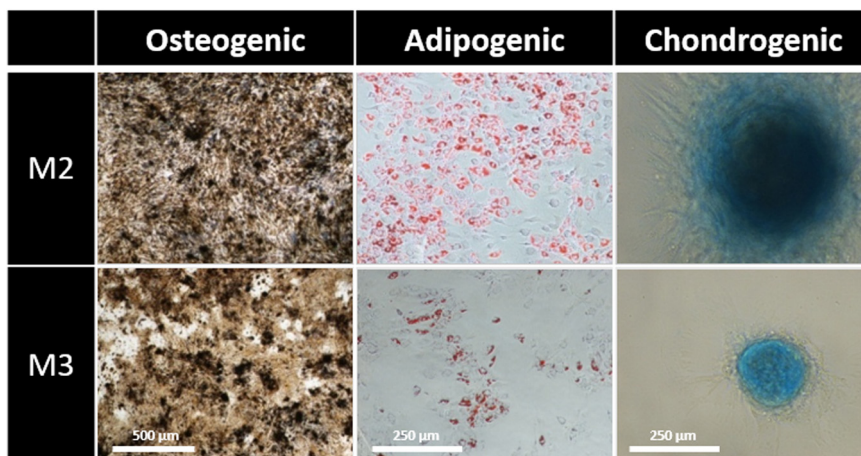


Fig. 5. Tri-lineage differentiation potential of two BM-hMSC lines using phase-contrast microscopy. Showing osteogenic differentiation by staining for alkaline phosphatase and calcium deposition, adipogenic differentiation by staining with Oil Red O and chondrogenic differentiation by staining with Alcian Blue. Images are representative of $n = 4$.

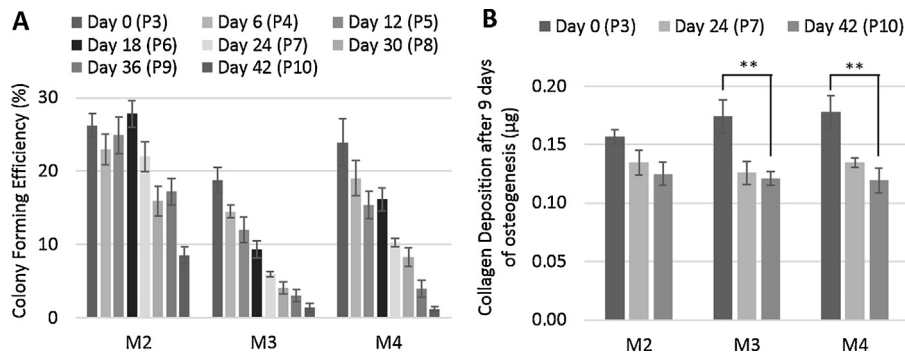


Fig. 6. Further characterization of three of the BM-hMSC lines throughout culture showing the differences in the colony forming unit fibroblast efficiency (A) and quantification of the collagen deposition after nine days under osteogenic conditions (B). (Data shows mean \pm SD, $n = 4$).

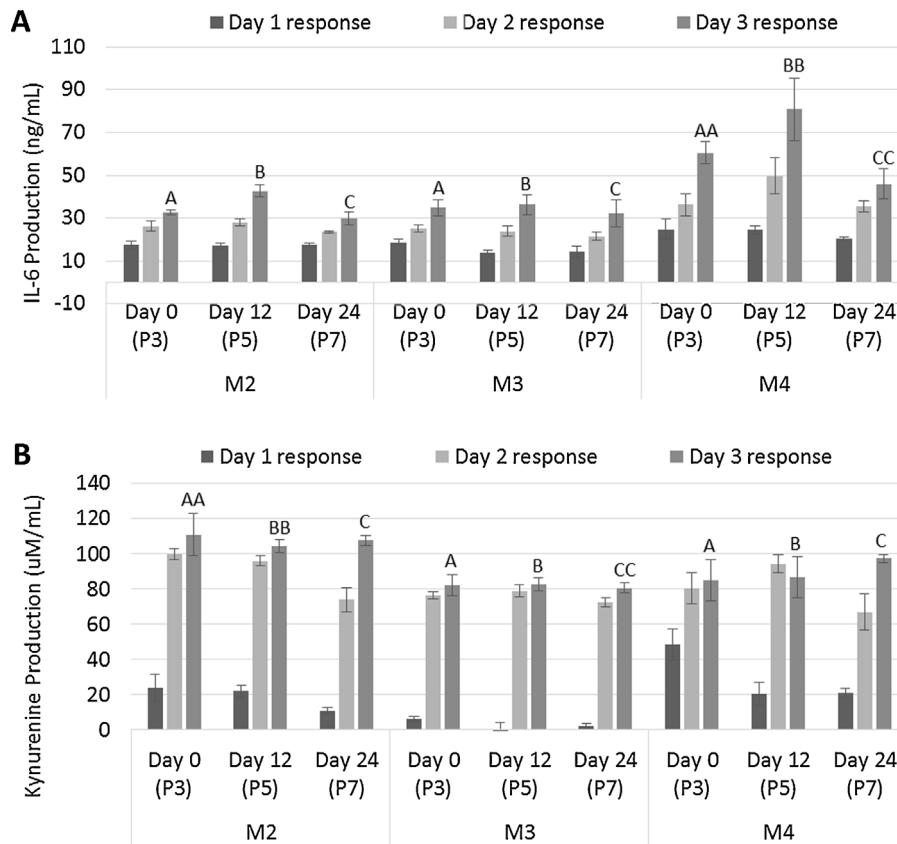


Fig. 7. Immunomodulatory characterization of three of the BM-hMSC lines throughout culture showing the differences in expression of IL-6 over three days (A) and the production of kynurenine over three days (B) in response to inflammatory stimuli. (Data shows mean \pm SD, $n = 4$). Statistical analysis shows single letter (no significance) double letter ($p < 0.01$) between donors. A – Day 0, B – Day 12, C – Day 24.

of dendritic cells and thus the activation of T-lymphocytes [22]. Fig. 7a shows the concentration of IL-6 over 72 h at passage 3, 5 and 7 when the BM-hMSCs were cultured with inflammatory stimuli. M4 secreted the most IL-6 through passage 3–5 ($p < 0.01$) with the maximum level of $80.8 \text{ ng/ml} \pm 8.4$ whereas M3 produced a maximum concentration of $36.3 \text{ ng/ml} \pm 3.65$. The rate of response for M4 was also quicker when compared to M2 and M3 as there was greater secretion of IL-6 within the first 24 and 48 h.

Kynurenine, a measure of tryptophan metabolism by indoleamine 2,3-dioxygenase (IDO), was also measured over the same period. IDO activity in BM-hMSCs has been shown to inhibit allogeneic T cell responses in mixed lymphocyte reactions [23]. In contrast to IL-6, M2 was found to produce the most kynurenine over each passage (Fig. 7b). The rate of kynurenine production can also be seen over the first 48 h, in all cell lines the rate of

response is reduced over the extended passage again suggesting a limit of culture-expanded BM-hMSCs for immunotherapy. Furthermore, as BM-hMSCs may only have a limited *in vivo* lifetime a faster response to the environment will be beneficial. Due to the putative multifaceted immunomodulatory mechanism of BM-hMSCs the critical mechanism of action (MoA) must be determined to then develop a robust and reproducible assay that can be used to screen input donor material. Whatever the MoA of BM-hMSCs for a specific clinical indication, it will be important to maintain consistency in these key attributes, demonstrating product understanding by maintaining a state of control over the product throughout the manufacturing process.

It has been shown previously that donor age and gender has an effect on the function of BM-hMSCs [24], particularly relating to their ability for immunoregulation *in vivo* [25]. With these intrin-

sis donor characteristics having such an effect on the functionality of the cell-based therapy products, it is important to understand how they will affect the final product. To maintain the consistency required within the process, it will likely be necessary to pre-screen donor material for both autologous and allogeneic products prior to the expansion process. By taking this approach it might be possible to reduce the impact of the variability in donor BM-hMSCs described, however, the ethical implication of pre-selecting patients for autologous cell-based therapies must be carefully considered.

3.4. Demonstrating comparability

Any changes to the manufacturing process during clinical development will require validation of the process to ensure it remains comparable before and after the change. Validating process changes will require significant time and resource, as well as the development of functional assays to demonstrate that there has been no change to the safety or function of the cell-based therapy product. In addition to making process changes, comparability must also be demonstrated if a cell-based therapy product is to be manufactured at multiple facilities [12]. Multisite manufacture has the advantage of reducing the capital cost for scaling out product manufacture to meet commercial demand as well as reducing the inherent risk of having a single manufacturing facility.

The process of demonstrating product comparability is not a trivial one and must go beyond the conventional in-process and product release characterization, requiring a large amount of process and product data during development to act as a stable foundation from which to demonstrate product comparability. At the core of this dataset is the establishment of a set of product CQAs linked to the product mechanism of action for a specific clinical indication. Demonstrating this level of product comparability for a process that is expanding cells from multiple donors will be challenging, as variability in the input material will reduce the process consistency. In addition to this, with a divergence in BM-hMSC growth as seen in Fig. 1b, increasing the number of product population doublings will make the process of demonstrating comparability more challenging still. Driving a consistent process will therefore be a logical first step towards developing these comparable processes, with reliable control strategies forming the basis for this consistency. Another aspect of ensuring consistency is sufficient control on the process input materials such as reagents, culture medium and disposables.

As can be seen from Figs. 6 and 7, the characteristics of each of these cell lines changes throughout the culture process. Depending on the target indication and the set of CQAs for the cell-based therapy product, this will make the demonstration of process comparability challenging for expanded cell-based therapy products. Each of the product characteristics monitored during this study have shown a reduction as the number of cell population doublings increases, a clear sign that reducing the expansion ratio of the product where possible will improve the chances of maintaining product functionality and demonstrating comparability. Understanding these process changes could be greatly improved by a detailed analysis of the metabolic activity of these products. Fig. 2 shows the net metabolite flux of glucose, lactate and ammonium, however, understanding the metabolic intermediates such as pyruvate would also aid in demonstrating that process changes have not inadvertently affected the product characteristics, an important aspect of process comparability.

3.5. Process analytical technology (PAT)

As highlighted above, defining and measuring relevant product characteristics forms a critical part of developing successful manu-

facturing processes for cell-based therapies. If these processes are to be successfully transferred to a scalable manufacturing platform, these parameters must not only be measured, but must be integrated into online monitoring and control strategies. Process analytical technology or PAT is a system for analyzing and controlling manufacturing processes through measurement of product attributes to ensure final product quality, proposed by the Food and Drug Administration (FDA) [26].

This process can be broken down into three distinctive steps [27]:

1. Understanding of the product quality attributes and how process parameters affect them.
2. Ability to analyze quality attributes and monitor critical process parameters.
3. Control of the critical process parameters to achieve consistent product quality.

It will therefore be desirable to begin to measure online parameters in order to develop control systems to ensure that the product characteristics described in section 3.3 remain consistent. These relevant process parameters are likely to include a combination of cell growth, medium temperature, pH, pO_2 and pCO_2 , which are commonplace in current biopharmaceutical production processes [28]. In addition to this, process parameters such as metabolite concentrations (glucose, lactate, ammonia and glutamine) can play a role in product understanding during the manufacturing process and should be controlled to ensure consistent product quality. The benefit of operating a production process under the guidance of PAT will likely be a reduction in product variability, which will reduce the likelihood of product batch failure. Having a detailed product understanding using online measurements also introduces the possibility for real-time release testing of product batches, which will reduce costs by reducing the quality test burden at the end of the process.

4. Conclusions

Characterizing input material from different BM-hMSC donors has allowed for an assessment of the effect of variation on developing cell-based therapy manufacturing processes. Identifying the divergent nature of the growth of multiple BM-hMSC donor lines has been identified as a potential issue for the development of both autologous and allogeneic processes where cell expansion is required. Furthermore, measuring multiple quality characteristics of these BM-hMSC lines throughout culture has demonstrated a reduction in quality as the population doubling level increases, which must be considered as these processes are scaled.

Developing manufacturing processes from multiple BM-hMSC donors will require an understanding of the effect of donor characteristics on expanded autologous and allogeneic cell-based therapy bioprocesses. Measuring informative product attributes that are characteristic of the desired therapeutic effect for each clinical indication will facilitate the development of consistent manufacturing processes and will play a key role in unlocking the value of demonstrating process comparability, allowing for any necessary process changes and multisite manufacturing models.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.bej.2015.06.018>

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