ARTICLE

BIOTECHNOLOGY and BIOENGINEERING

Closed System Isolation and Scalable Expansion of Human Placental Mesenchymal Stem Cells

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Received 27 September 2011; revision received 10 December 2011; accepted 19 December 2011

Published online 17 January 2012 in Wiley Online Library (wileyonlinelibrary.com). DOI 10.1002/bit.24425

ABSTRACT: Mesenchymal stem cells (MSC) are emerging as a leading cellular therapy for a number of diseases. However, for such treatments to become available as a routine therapeutic option, efficient and cost-effective means for industrial manufacture of MSC are required. At present, clinical grade MSC are manufactured through a process of manual cell culture in specialized cGMP facilities. This process is open, extremely labor intensive, costly, and impractical for anything more than a small number of patients. While it has been shown that MSC can be cultivated in stirred bioreactor systems using microcarriers, providing a route to process scale-up, the degree of numerical expansion achieved has generally been limited. Furthermore, little attention has been given to the issue of primary cell isolation from complex tissues such as placenta. In this article we describe the initial development of a closed process for bulk isolation of MSC from human placenta, and subsequent cultivation on microcarriers in scalable single-use bioreactor systems. Based on our initial data, we estimate that a single placenta may be sufficient to produce over 7,000 doses of therapeutic MSC using a large-scale process.

Biotechnol. Bioeng. 2012;109: 1817–1826.

KEYWORDS: mesenchymal stem cell; MSC; cell therapy; placenta; cell culture; scale-up

Introduction

Mesenchymal stem cells (MSC) are emerging as a leading cellular therapy for a number of diseases (Sensebé et al., 2010). Diverse applications range from improvement of cardiac function after severe acute myocardial infarction (Plewka et al., 2009; Flynn and O'Brien, 2011; Joggerst and Hatzopoulos, 2009) through to the treatment of inflammatory diseases [e.g., Crohn's disease and graft-versus-host disease (Dryden, 2009; Iyer et al., 2009; Kaplan et al., 2011)]. MSC also enhance wound healing and serve as a cell source for many tissue engineering applications, particularly those targeting skeletal tissues (Panetta et al., 2009; Richardson et al., 2010; Runyan and Taylor, 2010). However, for these treatments to become available as a routine therapeutic option, efficient and cost-effective means for industrial manufacture of MSC are required.

The starting cells used to manufacture an MSC product can be isolated from a variety of tissues. The most widely researched source of MSC is bone marrow (Charbord, 2010; Mosna et al., 2010), while adipose (Mosna et al., 2010; Wilson et al., 2011), placenta (Barlow et al., 2008; Li et al., 2010), and umbilical cord (Bieback and Klüter, 2007; Jäger et al., 2009) derived MSC are increasing in usage. We have previously shown that human placenta is a rich source of MSC, and such cells are highly similar to bone marrow derived MSC (Barlow et al., 2008). In contrast to bone marrow harvest, however, placenta is available in abundance at no extra risk to the donor (placenta is collected after the safe delivery of the child). Not only does placenta present an essentially unlimited supply of readily accessible starting material, the large size of individual placentas (typically 500–750 g) leads to substantial numbers of MSC per individual donor.

Regardless of source, it is necessary to propagate MSC in vitro to increase cell numbers, a process referred to as ex vivo cell expansion. The standard method of expansion relies on cultivation of donor-derived MSC in plastic tissue culture flasks. As the cells increase in number, cultures are manually

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passaged by enzymatic release from the plastic surface, and then re-seeded into a larger number of flasks for further culture. In this manner, the available growth area is progressively increased to accommodate growth of the MSC. For routine commercial production, this manual approach is inappropriate due to the open nature of manual processing, associated safety risk, high labor costs, and requirement for expensive infrastructure.

Scale-up and automation of adherent cell culture is not a new challenge, and was effectively dealt with decades ago by the biopharmaceutical industry for production of vaccines and therapeutic proteins. The most common solution is the use of microcarriers (GE Healthcare, 2005), small particles typically several hundred micrometers in diameter, to enable cultivation in controlled bioreactor systems.

A number of published reports describe cultivation of MSC from various tissues and species on microcarriers (Eibes et al., 2010; Frauenschuh et al., 2007; Hewitt et al., 2011; Malda and Frondoza, 2006; Sart et al., 2009; Schop et al., 2008; Yang et al., 2007). While these studies have demonstrated that microcarrier cultivation of MSC is feasible, and that the resulting cell populations were equivalent to flask-expanded controls, typically limited cell expansion was achieved and processing of the donor tissue was not addressed. Few of these studies were performed using human cells, and none of the processes described were suitable for manufacture of a human therapeutic product on a routine basis. In a significant advance over these earlier reports, Santos et al. (2011) recently described a xeno-free micocarrier-based approach, achieving expansions of 18- and 16-fold over 14 days for bone marrow and adipose tissue-derived MSC, respectively. Seeding efficiencies (and hence overall process efficiency) were, however, reported to be low, and it has been our experience that the medium and substrate system employed (StemPRO/CellStart) does not support the cultivation of human placental MSC (hpMSC).

To facilitate clinical use of hpMSC, we are developing a closed and scalable process for routine manufacture. In this article we describe the initial development of a closed process for both the isolation of MSC from human placenta and their subsequent expansion in a scalable single-use bioreactor system.

Materials and Methods

Cell Isolation

Term placenta were collected following routine caesarean section with informed consent and institutional ethical approval. For manual processing, cells were isolated according to the method described by Barlow et al. (2008) and Brooke et al. (2009). Briefly, the umbilical cord and external membranes were removed, followed by dissection of approximately 5 cm^3 pieces of placental tissue. These tissue pieces were washed in Hanks balanced salt solution (HBSS; Invitrogen, Mt. Waverly, Victoria, Australia) and further dissected into small fragments. Fragments were then subjected to enzymatic digestions with 100 U/mL collagenase I (Worthington Biochemical Corp., Lakewood, New Jersey, USA) and 100 U/mL DNase I (Roche Diagnostics Australia Pty. Ltd., Castle Hill, New South Wales, Australia) in low glucose Dulbecco's modified Eagles medium (LGDMEM; Invitrogen) for 2 h at 37°C. Following digestion, remaining solid tissue was removed from the slurry by a combination of pulse centrifugation and passage through a 70 μm cell strainer. The cell suspension was then subject to density gradient centrifugation using Ficoll-Paque (1.073 g/mL; GE Heatlthcare Bio-Sciences Pty. Ltd., Rydalmere, New South Wales, Australia) for 20 min at 535 rcf. The interfacial layer was recovered, rinsed in HBSS, and resuspended in LGDMEM for counting by trypan blue exclusion and subsequent cultivation.

In the semi-automated approach, 40 g pieces of intact placenta (i.e., including the membranes) were placed into sterile blender bags, to each of which 80 mL of enzyme digest solution comprising 100 U/mL collagenase I, 2.5 U/mL dispase (Invitrogen), and 100 U/mL DNase in LGDMEM were added. Using a paddle blender (Masticator, IUL Instruments, Barcelona, Spain), the bag contents were intermittently blended for 2 min followed by 18 min incubation at 37°C, for a total of 1 h. The resulting cell slurry was passed through 500 and 280 μm screens to remove undigested fragments. The resulting slurry was then either pelleted and red blood cells removed by ammonium chloride lysis prior to cell enumeration by trypan blue exclusion, or taken through to culture.

Flask Culture

Manually isolated cells were seeded into Nunc tissue culture flasks at a density of 2,500 cells/cm^2 in LGDMEM supplemented with 50 μg/mL gentamicin (Pharmacia, Pfizer Asutralia, West Ryde, New South Wales, Australia) and 20% fetal bovine serum (FBS; Invitrogen). For cells isolated by the semi-automated method where ammonium chloride lysis was employed, the same approach was taken. Where no lysis step was used, the cell slurry was diluted in medium to match the above formulation and seeded directly into flasks. In both cases, medium was replaced 48–72 h later to remove non-adherent cells. At 90–95% confluence, cultures were rinsed with HBSS and dissociated using TrypLE select (Invitrogen), enumerated by trypan blue exclusion and reseeded at 2,500 cells/cm^2. All cultures were undertaken at 37°C in a humidified 5% CO_2 in air atmosphere using standard cell culture incubators.

Small-Scale Microcarrier Culture

A range of microcarriers were investigated for suitability in the cultivation of hpMSC (Table I), and various seeding

Table I. Microcarriers screened for hpMSC culture.

Microcarrier	Manufacturer	Material	Surface
I			
Cytodex 1	GE Healthcare	Dextran	DEAE groups
Cytodex 3	GE Healthcare	Dextran	Gelatin
Glass	Sigma–Aldrich	Glass	Unmodified
CultiSpher-S	Percell Biolytica AB	Porous gelatin	Unmodified
FACT	Solohill Engineering, Inc.	Polystyrene	Cationic type I porcine collagen
ProNetcin	Solohill Engineering, Inc.	Polystyrene	Recombinant RGD peptide
Collagen	Solohill Engineering, Inc.	Polystyrene	Type I porcine collagen
II			
Hillex II	Solohill Engineering, Inc.	Modified polystyrene	Cationic trimethyl ammonium
MicroHex	Nalgene Nunc International	Polystyrene	Nunc Δ
Plastic	Solohill Engineering, Inc.	Polystyrene	Unmodified
PlasticPlus	Solohill Engineering, Inc.	Polystyrene	Cationic
Cytopore 1	GE Healthcare	Porous cellulose	DEAE groups
Cytopore 2	GE Healthcare	Porous cellulose	DEAE groups

(I) Initial screening with quantitative assessment of seeding efficiency. (II) Introduced in later experiments, qualitative assessment of seeding efficiency. DEAE group refers to *N*,*N*-diethylaminoethyl.

and culture formats were employed. These included static dishes/flasks, static tubes, shake tubes, shake flasks, and spinner flasks. The most consistent performance was ultimately achieved by overnight seeding under static conditions in a minimum amount of complete medium. After 6–7 days culture in the same medium as above, microcarriers were collected into sterile tubes, washed with HBSS and the cells released by enzymatic treatment. Following further washing in HBSS to remove enzymes, cells could be reseeded on to fresh microcarriers.

Wave-Type Bioreactor Microcarrier Culture

CultiSpher-S microcarriers were introduced to 2 L CultiBag (Sartorius-Stedim) or Cellbag (Wave, GE Life Healthcare) culture bags in the minimum possible volume of medium required to facilitate transfer. Cells were then similarly introduced in a minimal volume of medium, and the bag gently rocked several times in order to distribute the cells amongst the microcarriers. Following overnight seeding without rocking, medium volumes were increased to 500 mL and rocking commenced. As using a standard atmosphere of 5% CO_2 in air for such cultures gave poor results, a 5% O_2/ 5% CO_2 mix was ultimately used.

Quantification of Cell Seeding Efficiency

Following incubation of cells with microcarriers for 18 h, culture medium was collected by decanting. Microcarriers and the culture vessel were rinsed twice and again decanted to maximize recovery of any unattached cells. The decanted volumes were combined, centrifuged at 300 rcf for 10 min, and assayed using a CyQUANT cell quantification assay kit (Invitrogen) as per the manufacturers' instruction. To determine seeding efficiency, values for the decanted cells were compared against triplicate samples taken at the time of culture initiation (i.e., 100%).

Immunofluoresence Microscopy

Cell-bearing microcarriers were labeled with Mitotracker Orange (Invitrogen), fixed in a 4% paraformaldehydes solution for 10 min and rinsed with PBS. Samples were subsequently permeablized with 0.1% Triton X-100 and labeled with Alex Fluor 647 conjugated phalloidin (Invitrogen) and DAPI (Invitrogen). Images stacks were captured using a Ziess LSM 510 Meta and flattened to enable in-focus visualization of spherical microcarrier surfaces.

Flow Cytometry

Following dissociation using TrypLE select, cells were washed and incubated for 20 min at room temperature with either CD45-FITC, CD44-PE, and CD90-APC, or CD73-PE, CD105-APC, or CD-146-FITC (BD Australia, North Ryde, New South Wales, Australia). Matched isotype controls were used, and dead cells were detected by staining with 7AAD (Invitrogen). Samples were analyzed using a BD LSR II flow cytometer.

Mesodermal Differentiation

Osteogenic, chondrogenic, and adipogenic differential potential were qualitatively assessed using standard methods (e.g., Barlow et al., 2008). Osteogenic differentiation was induced over 3 weeks by cultivation in high glucose DMEM (HGDMEM)supplemented with 10% FBS (Invitrogen), 0.1 μM dexamethasone (Sigma–Aldrich), 50 μM L-ascorbic acid-2-phosphate (Sigma–Aldrich), 10 mM β-glycerol phosphate disodium salt pentahydrate (Sigma–Aldrich), and 0.3 mM inorganic phosphate (Sigma–Aldrich). Calcium deposits were visualized by staining with AlizarinRed S (Sigma–Aldrich). Chondrogenic differentiation was induced in pellet cultures initiated from 5×10^5 MSC and cultured for 3 weeks in HGDMEM supplemented with

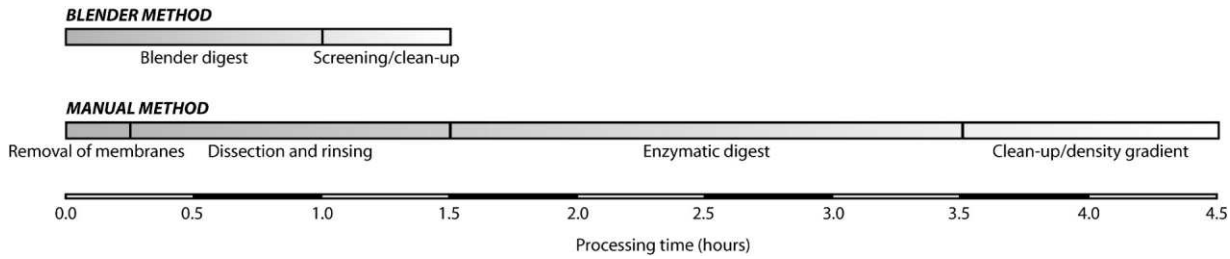

Figure 1. Timelines for blender-based and manual processing of human placenta. While the blender-based approach is capable of processing an entire placenta in 1.5 h, 4–5 h are required to process only 50 g (<1/10th of the total) of tissue using the manual method.

0.1 μM dexamethasone, 1 mM sodium pyruvate (Sigma–Aldrich), 50 μM L-ascorbic acid-2-phosphate, 35 mM L-proline (Sigma–Aldrich), 10 ng/mL TGF-β3 (Peprotech) and 50 mg/mL ITS Premix (BS Biosciences). Glycosaminglycans were visualized by staining frozen sections of the cell pellets with Alcian Blue (Sigma–Aldrich). Adipogenic differentiation was induced over 3 weeks by cultivation in HGDMEM supplemented with 1 μM dexamethasone, 5 μg/mL insulin (Sigma–Aldrich), 60 μM indomethacin (Sigma–Aldrich), and 0.5 mM 3-isobutyl-1-methylxanthine (IBMX; Sigma–Aldrich). Lipid droplets were visualized by staining with Oil Red O (Sigma–Aldrich).

Results

Development of Semi-Automated Isolation Process

We have employed a non-contact paddle blender (e.g., IUL Instruments Masticator, Seward Stomacher) for semi-automated processing of placenta. In these blenders, the tissue is placed into a sterile sample bag which is then subjected to a crushing action by alternating compression of the contents by mechanical paddles. In the case of placenta, an enzyme digest solution is added to the bag prior to blending in order to facilitate the release of cells from tissue fragments.

We undertook a head-to-head comparison of an existing manual isolation protocol (Barlow et al., 2008; Brooke et al., 2009) and a semi-automated process using a paddle blender. Although viability following isolation by the semi-automated process resulted in lower cell viabilities (82.4% vs. 96.6%; $P < 0.01$, Students t-test; $n = 5$), this reflected differences in the clean-up step following digestion. A more appropriate comparison can made on the basis of cell yields following four serial passages in tissue culture flasks. Final yields of 5.19×10^9 and 6.60×10^9 cells/g were obtained for the semi-automated and manual process respectively ($P = 0.675$, Students t-test; $n = 5$). While the two processes give equivalent yields of MSC per gram of placenta, the semi-automated process provided significant time savings. Using this approach, placental tissue can be processed within 1–1.5 h, and blenders with capacities of 3.5 L (sufficient for entire placenta) are available. Using the manual process it takes 4–5 h to process only 40–50 g (<1/10th of the total) of tissue (Fig. 1).

In the semi-automated process it is not necessary to remove the external membranes (undigested material is removed by screening) and consequently all steps subsequent to placement of the placenta in the bag and sealing can be undertaken in a closed fashion. With an appropriately designed bag, rinsing and digest solutions can be introduced aseptically, and post-digest screening to remove debris can be achieved in-line. Although we have not yet integrated such an approach, an in-line filtration based concentration step would enable full automation and process closure.

Cell Expansion on Microcarriers

In order to produce sufficient cells for therapeutic purposes, it is usual practice to expand isolated MSC (regardless of tissue origin) in tissue culture using flasks, roller bottles, or cell factories (essentially multi-layered flasks). Such approaches do not scale well, and automation requires expensive robotics. The most common industrial approach to scale-up of adherent cell culture is the use of microcarriers, facilitating cultivation in standard bioreactor systems.

Seeding

Microcarriers are available in a range of materials, geometries, and architectures. In order to translate hpMSC cultivation to a microcarrier format, we initially screened a panel of seven microcarriers (Table I-I). We first assessed the performance of these microcarriers with respect to seeding efficiency. Unmodified glass microcarriers performed poorly and were thus rejected as a candidate. For the remaining six microcarrier types we were able to achieve seeding efficiencies of >75% in all cases (Fig. 2A) by maximizing the interaction between cells and microcarriers (i.e., seeding in a minimal volume of medium under static

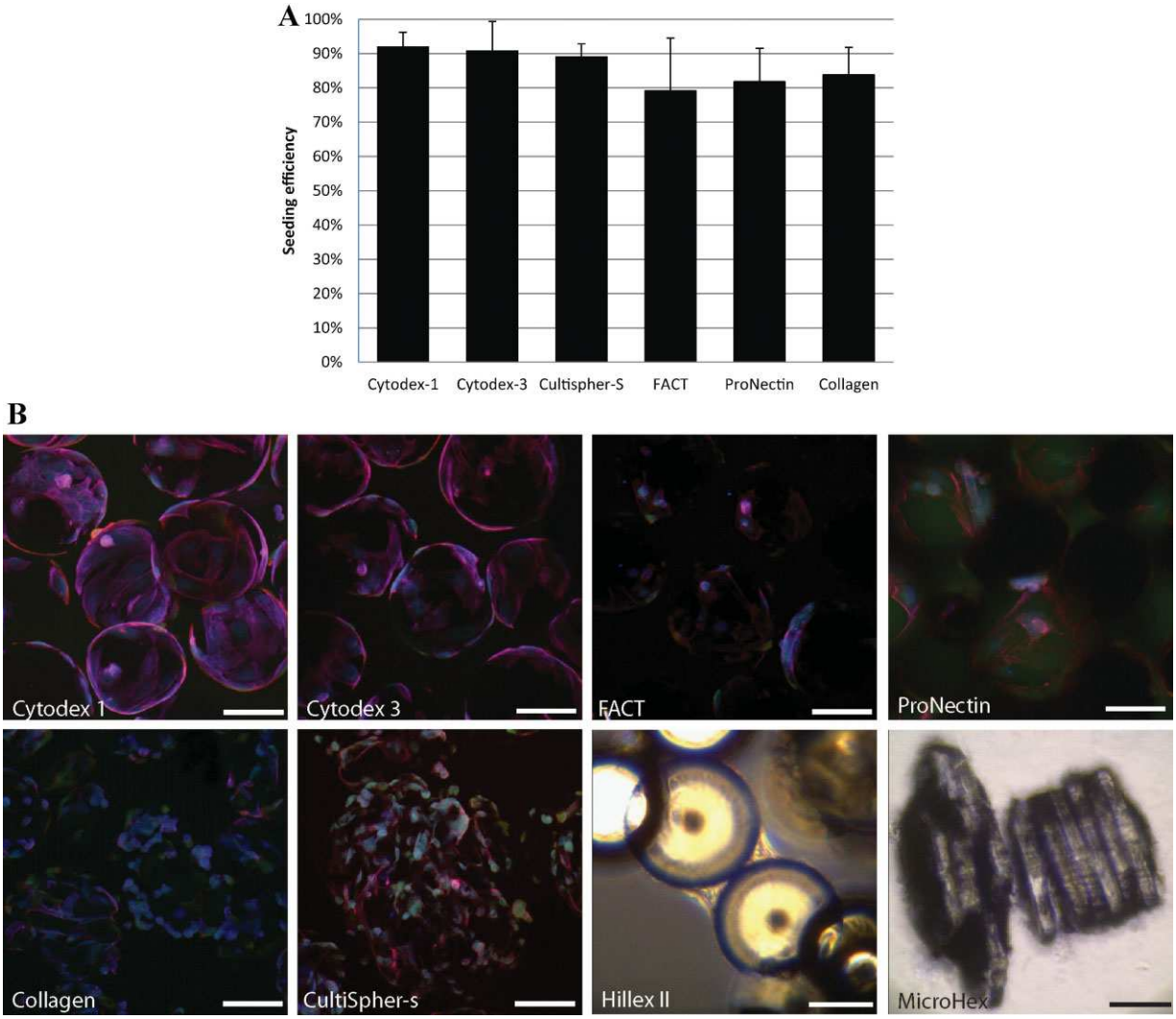

Figure 2. **A**: Seeding efficiency of hpMSC on a selection of commercially available microcarriers ($n = 4$; mean ± SD). **B**: Morphology of hpMSC growing on a selection of commercially available microcarriers. Fluorescent images: Magenta = actin, blue = nuclei, green = mitochondria (the diffuse green coloring of FACT cultures is predominantly due to autofluorescence of the microcarriers). MicroHex cells are stained with MTT giving a blue/black color. Scale bars = 100 μm. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/bit]

conditions). Seeding efficiencies of ≥90% were achieved for Cytodex 1 and 3, and CultiSpher-S.

Growth

Following quantification of seeding efficiency, we undertook a visual of assessment of hpMSC growing on microcarriers with respect to morphology and coverage (Fig. 2B, fluorescence images). Cytodex 1 and 3 cultures exhibited a flattened and spread morphology as would typically be observed in monolayer cultures, with good coverage of the microcarriers. While FACT cultures were also flattened, coverage was comparatively poor. ProNectin cultures also exhibited a flattened and spread morphology, while the microcarriers themselves exhibited a degree of autofluoresence. In contrast, cells in both collagen and CultiSpher-S cultures were not flattened, but rather appeared to be predominantly loosely attached and balled-up.

At this point several additional microcarrier types (Table I-II) were acquired and assessed qualitatively. Hillex II microcarriers performed extremely well with regards to seeding, with very few cells visible in suspension after only 1 h. While it was not possible to observe these cultures by fluorescent microscopy due to the binding properties of Hillex II (dyes/antibodies bind directly to the microcarriers), cells were clearly visible growing between microcarriers (Fig. 2B, phase contrast images). MicroHex carriers (hexagonal flakes of Nunc tissue culture plastic) gave qualitatively similar seeding performance as for tissue culture flasks. While in these cultures the cells also grew well,

the geometry resulted in stacking of the microcarriers (Fig. 2B). Seeding on Solohill Plastic and PlasticPlus microcarriers was evidently poor from the large numbers of cells that could be observed in suspension following overnight seeding.

Expansion

Having assessed seeding performance and morphology, we next assessed expansion over 7 days using a variety of culture formats and protocols. These included static flasks, rocked flasks, static tubes, shake tubes, shake flasks, and spinner flasks. As microcarrier performance differed depending on format, and it was not feasible to optimize each format for all microcarriers, we compared the maximum observed expansions regardless of format (Fig. 3). CultiSpher-S achieved the highest degree of expansion (~15-fold) followed by MicroHex, collagen, Cytodex 3, and Hillex II. Despite good coverage of cells growing on Cytodex 1 microcarriers, the apparent expansion was poor. While hpMSC both attach and grow extremely well on Cytodex 1, recovery of the cells was problematic. After testing multiple enzymatic treatments, cold shock, and treatment with lignocane (GE Healthcare, 2005), we failed to identify a protocol consistently resulting in good recovery of cells from Cytodex 1. While recovery from Cytodex 3 was better, performance was inconsistent. As a consequence, both Cytodex 1 and 3 were not pursued further. While MicroHex carriers performed well with respect to seeding and expansion, we observed black particulate material in the packaged product and in cultures. MicroHex carriers were also prone to fragmentation in some culture formats. As the particulate matter and fragments may be problematic during product harvest and clean-up, MicroHex was not further pursued for scale up of hpMSC cultivation. We also tested Cytopore 1 and 2 microcarriers (data not shown), neither giving sufficient cell yields in our hands to justify further investigation.

To verify expansion performance on CultiSpher-S, Collagen, and Hillex II microcarriers, triplicate cultures at seeding densities of 5, 10, and 15 cells/microcarrier in static flasks were undertaken (Fig. 4A). CultiSpher-S gave the best result (14.9 ± 1.2-fold expansion at 5 cells/carrier), followed by Hillex II (9.05 ± 1.08-fold at 5 cells/carrier). Collagen microcarriers performed poorly (3.58 ± 0.27 at 5 cells/carrier). While collagen carriers do in fact perform well under certain conditions (as indicated in Fig. 2), it appears that cell attachment is comparatively weak (as suggested by the morphology of Fig. 2B and observation during handling) and the cells are prone to detachment when cultures are handled.

A clear correlation between seeding density and expansion was apparent. This is not unexpected as while microcarriers enable very high surface area per unit culture volume, the amount of surface available per microcarrier remains limited. To further illustrate this principle, we undertook duplicate cultures on CultiSpher-S microcarriers

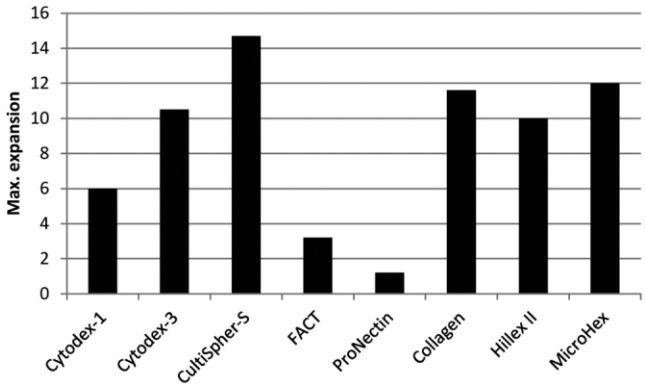

Figure 3. Maximum observed expansion of hpMSC over 7 days. As microcarrier performance may vary according to culture format, each was tested in static flasks, rocked flasks, static tubes, shake tubes, shake flasks, and spinner flasks. The maximum expansion obtained from any one of these methods is presented, giving a relative indication of performance without the need for time-consuming individual optimization for each microcarrier type.

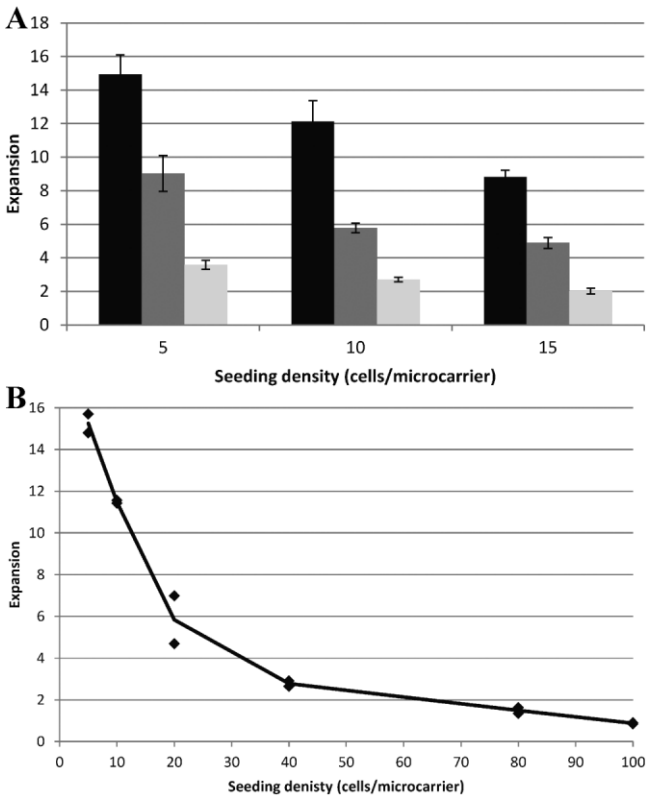

Figure 4. **A**: Cell expansion after 7 days for hpMSC grown on CultiSpher-S (black), Hillex II (dark gray), and collagen (light gray) microcarriers (mean ± SD, $n = 3$). **B**: Seeding density dependent expansion of hpMSC on CultiSpher-S microcarriers ($n = 2$, each data point displayed).

at seeding densities of 5, 10, 20, 40, 80, and 100 cells/microcarrier (Fig. 4B). While seeding densities below 3 cells/carrier did on occasion outperform those at 5 cells/carrier (data not shown), we found these to be inconsistent.

Preliminary Scale-Up

In order to facilitate large-scale culture of hpMSC, we undertook cultures on CultiSpher-S microcarriers in wave-type bioreactors. Initially these cultures performed poorly (data not shown), even when first initiated in static flasks and subsequently transferred to bioreactor bags. Control cultures using media pre-incubated overnight in bioreactor bags indicated that this was not due to any cytotoxicity associated with the bag materials (data not shown). We suspected the problem related to agitation and over oxygenation of low cell density cultures, as previously observed for hematopoietic stem cell cultures (Timmins et al., 2009, 2011). In subsequent cultures where O_2 levels were reduced to 5%, expansions of 15.7- and 16.3-fold were obtained in 0.5 L cultures over 7 days.

Using existing commercial bioprocess technologies, it is possible to integrate our blender-based isolation method with subsequent cell expansion in a bioreactor (Fig. 5). A key feature of this approach is that passaging in situ (i.e., directly in the culture bag) is rendered relatively simple by the soluble nature of CultiSpher-S microcarriers. Utilizing built in perfusion membranes within the culture bags, medium is removed, cell-laden microcarriers washed with buffer, CultiSpher-S enzymatically degraded, cells washed with buffer, and fresh medium and microcarriers introduced aseptically.

hpMSC Phenotype—An Equivalent Cell Product

In order to determine if semi-automated isolation using a paddle blender and microcarrier cultivation on CultiSpher-S resulted in gross phenotypic changes, we characterized mesodermal differentiation potential and expression of cell surface markers.

Independent triplicate cultures of cells isolated using a paddle blender and expanded over four serial passages on CultiSpher-S were compared to cells isolated manually and expanded in tissue culture flasks for expression of the cell surface markers CD73, CD90, CD105, CD44, CD146, and CD45 (Fig. 6A). No statistically significant differences in median fluorescence intensities were observed, with $P = 0.87$, 0.16, 0.99, 0.41, 0.40, and 0.59, respectively (2-sided, 2-sample, Students t-test).

In mesodermal differentiation cultures (Fig. 6B), blender-isolated and CultiSpher-S expanded hpMSC exhibited strong potential for osteoblast and chondrocyte differentiation, with extensive calcium deposits in the former, and glycosaminoglycan expression in pellet cultures. Accumulation of lipid droplets was detected in adipogenic cultures; however, as previously demonstrated for hpMSC (Barlow et al., 2008), this was not as pronounced as with bone marrow-derived MSC. For all three lineages, differentiation potential was qualitatively similar to that routinely observed in our laboratories for manually isolated and flask-expanded hpMSC.

Discussion

MSC present many exciting opportunities for treatment of a diversity of diseases. In particular, the immunological

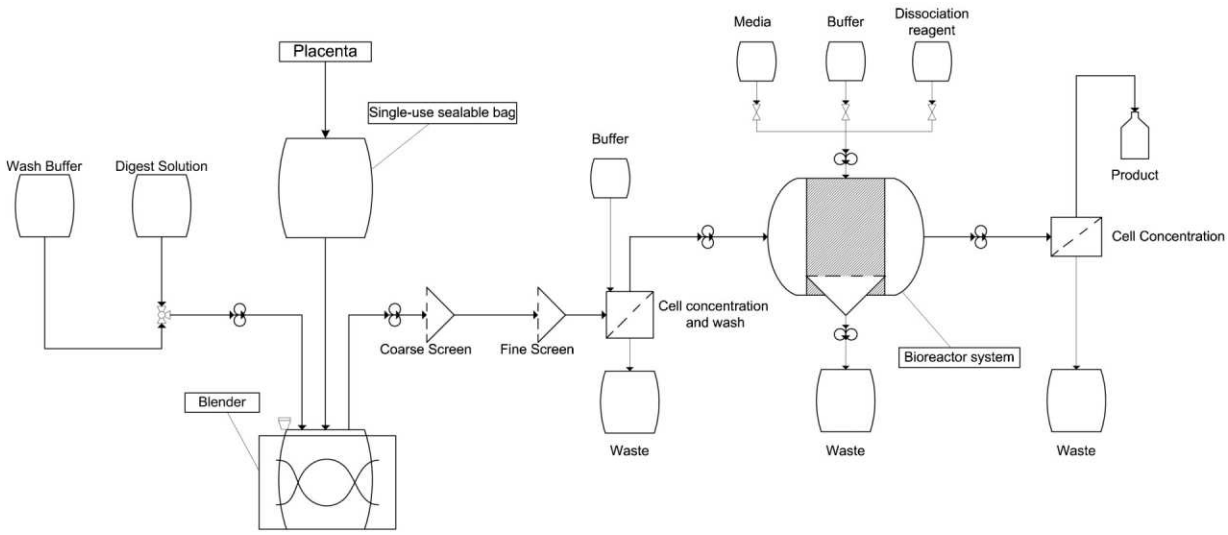

Figure 5. Representation of an integrated and fully closed process for the manufacture of clinical grade hpMSC.

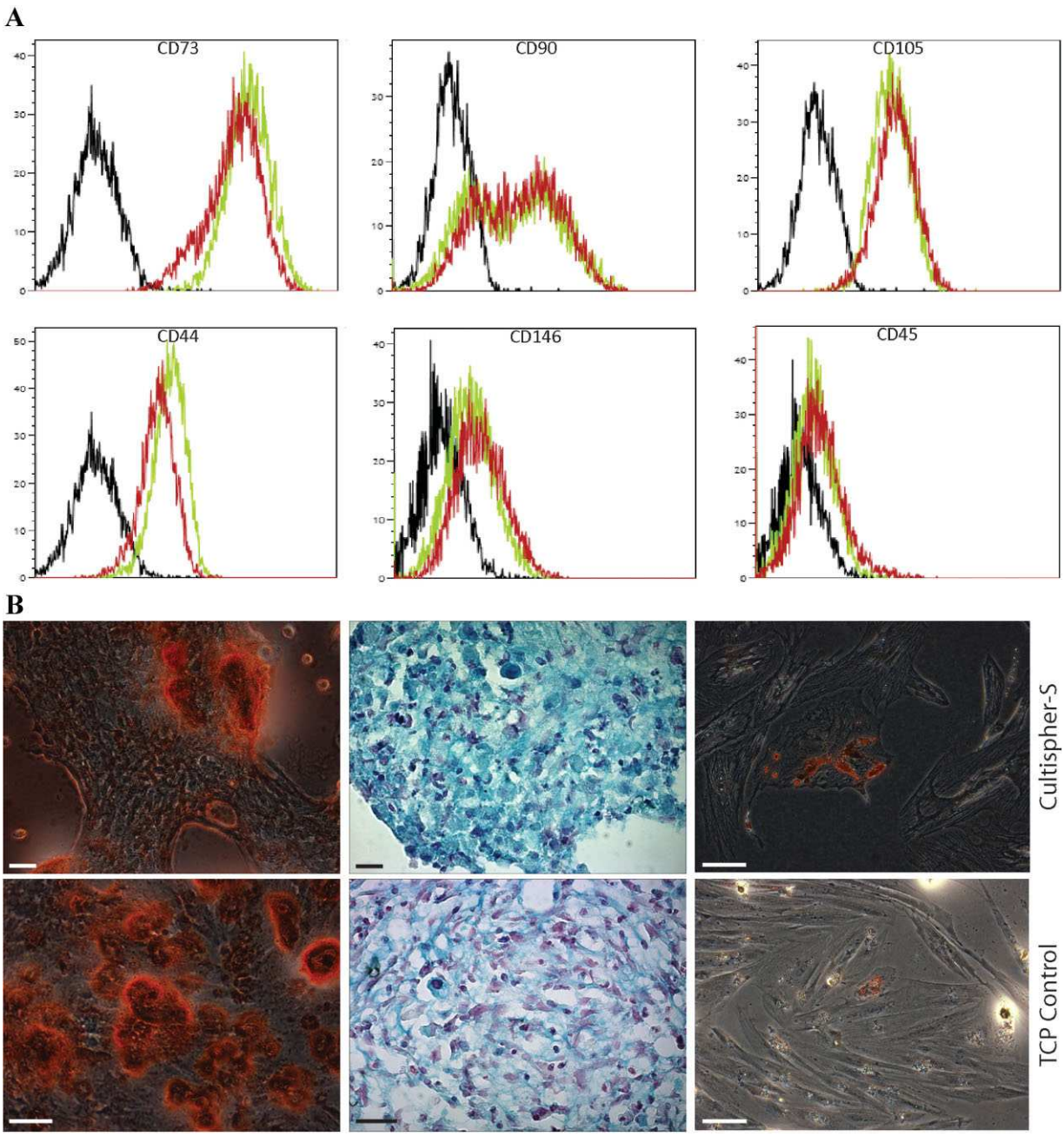

Figure 6. **A**: Representative histograms of cell surface marker expression after four serial passages in tissue culture flasks (red) or on CultiSpher-S (green). No statistically significant differences in median fluorescence intensity were observed, black = isotype control. **B**: Osteogneic (left, Alizarin Red), chondrogenic (centre, Alcian Blue), and adipogenic (right, Oil Red O) differentiation of hpMSC after blender isolation and cultivation on CultiSpher-S for four passages compared to TCP controls. Both sets of cultures appeared qualitatively similar. Scale bar = 50 μm. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/bit]

behavior of MSC is permissive of numerous allogeneic applications without the need for donor–recipient matching, enabling the possibility of a routine ''off-the-shelf'' cellular product readily available to clinicians. However, appropriate methods for the manufacture of such a therapeutic MSC product have not yet been described.

Placenta is an attractive source of starting material for such an off-the-shelf allogeneic cellular product due to its abundance and lack of risk to the maternal donors. However, isolation of cells from the placenta is time-consuming, and current manual methods are relatively high risk due to the open nature of the process. This approach is also limited with respect to the amount of material that can realistically be processed per technician, leading either to excessive labor costs (and associated infrastructure) or wastage of donor material.

During manual isolation, dissection of the placenta is in practice a size-reduction step that increases tissue exposure to the enzymatic digest solution. Clearly this outcome might be achieved more easily using a mincing or blending action. Apparatus for this purpose typically employs steel blades or augers, which directly contact the material being processed. For the purpose of processing placenta, these components would either need to be readily cleanable and sterilizable (increasing complexity and reducing throughput) or disposable (requiring appropriate design and fabrication at low cost). An alternative blending approach commonly used in the preparation of food samples for microbiological analysis, is the non-contact reciprocating paddle blender. In these devices the material to be blended is aseptically transferred to a sterile bag which is then closed. The bag (and sample) is then subject to compression applied to the external surface of the bag using paddles. By using two paddles with an alternating action, the sample is progressively crushed and simultaneously mixed with any fluids in the bag. By controlling the duration, speed, and pressure of the paddle action, different blending outcomes can be achieved. In this process the only component of the blender in direct contact with the sample material (e.g., placenta) is a low cost disposable plastic bag.

An immediate concern with such an approach is the possibility of cell damage as a consequence of the harsh environment generated by blending. However, due to differences in the digest material obtained immediately following processing by either the manual or paddle blender methods, direct comparisons of cell viability following isolation are of limited value. Whereas the manual process results in a relatively "clean" suspension of single cells and small cell clumps with few RBC, the blender approach results in a cell slurry containing larger tissue fragments and large numbers of RBC, both of which interfere with the determination of cell viability and yield. To eliminate RBC and facilitate determination of viability by trypan blue exclusion, we used ammonium chloride lysis. While the viability of blender-processed cells was only 82.4% compared to 96.6% for the manual process, we believe that this was more a reflection of differences in the sample clean-up rather than any substantial increase in damage incurred by the paddle blender. Indeed, if the blending action was the cause of significant cell damage, we would anticipate much lower viability.

Due to the different natures of the material obtained following digest by the two methods, a more appropriate measure of effectiveness is the number of hpMSC ultimately obtained from a given quantity of tissue. After four serial passages in tissue culture flasks, no statistical difference in cell yields per gram of starting material was observed. During culture following blending, RBC were removed through media exchange (as is usual for the removal of non-adherent cells), while tissue fragments not removed by screening were either removed in the same manner, or attached to the culture surface and gave rise to cell outgrowths. Subsequent to the first passage, cells obtained by either method behaved similarly and were indistinguishable when observed directly.

Our paddle blender-based approach to tissue digestion provides a platform for single-use closed processing of an entire placenta in a relatively short period of only 1–1.5 h. This will provide substantial advantages with regards to cost, product safety, and effective cell yield per donation. To fully realize these benefits however, a subsequent process for efficient expansion of MSC is required.

The use of microcarriers for adherent cell culture in bioreactor systems is a well-established technique. It has been demonstrated by several groups using cells derived from different tissues and species that MSC can be expanded on microcarriers without apparent changes in phenotype relative to standard flask based cultures (Eibes et al., 2010; Frauenschuh et al., 2007; Hewitt et al., 2011; Malda and Frondoza, 2006; Sart et al., 2009; Schop et al., 2008; Yang et al., 2007). Santos et al. (2011) recently described a xeno-free method for microcarrier-based expansion of bone marrow- and adipose tissue-derived MSC. This approach achieved expansions of 18- and 16-fold, respectively, over 14 days, a significant improvement over previous reports. It has been our experience that the medium/substrate system employed by Santos et al. does not support maintenance or growth hpMSC (data not shown). Furthermore, the use of plastic (or other insoluble material) microcarriers can complicate and reduce cell recovery at harvest due to inefficiencies in the separation of the cell suspension from the mirocarriers.

Although currently reliant on serum-based medium for hpMSC expansion, the process we describe achieves similar levels of expansion to that of Santos et al. (2011) in only 7 days. Furthermore, culture manipulations are facilitated by the ability to fully dissolve CultiSpher-S microcarriers, and our approach achieves substantially higher seeding efficiencies [$>$90% compared to $\sim$22% in xeno-free conditions (Santos et al., 2011)], which ultimately translates to higher cell yields for a given quantity of starting material. More significantly perhaps, the two approaches are complementary. Upon identifying an appropriate medium/substrate system for serum/xeno-free cultivation of hpMSC, it should be relatively straightforward to adapt this system for large-scale cultivation using our process.

Our data indicate that neither our blender-based approach to isolation, nor subsequent cultivation on CultiSpher-S alter cell phenotype relative to standard flask expansion. More detailed characterizations, including functional potency assays, are ultimately required. At this early stage, however, there is no indication that cultivation of hpMSC on CultiSpher-S is detrimental to the final cell product.

Although simple, our isolation and culture methods are highly effective. Our data indicate that a single 500 g placenta is sufficient to produce enough hpMSC for 2×70 kg patients at a dose rate of 5×10^6 cells/kg, at the end of passage 1. Assuming no decrease in expansion per passage (as for flask cultures), cultivation over four serial

passages has the potential to produce more than 7,000 such doses per placenta in large-scale bioreactor systems. This would have significant benefits with respect to product validation and consistency, and reduce the cost associated with procuring and screening source material to a triviality.

We have demonstrated the initial steps to development of a simple closed process for automated isolation of MSC from whole human placenta, and subsequent cultivation in scalable single-use bioreactor systems. With further optimization it is reasonable to expect increases in both process efficiency and product yield, in parallel to the evolution of a fully closed and automated manufacturing solution for efficient and low cost production of an off-the-shelf allogeneic hpMSC therapy.

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