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The disc-shaped microcarriers: A new tool for increasing harvesting of adipose-derived mesenchymal stromal cells

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ABSTRACT

Human adipose-derived mesenchymal stromal cells (A-MSC) are promising tools for regenerative medicine, but their in vitro amplification before administration is still an issue. Microcarriers emerge as a novel approach for A-MSC expansion in bioreactors. Here, microcarriers with different shapes and functionalizations were compared according to their efficiency for A-MSC expansion. In particular, novel disc shape microcarriers were compared to the commonly used spherical microcarriers. This geometry increases their specific surface while presenting a flat surface, close to monolayer culture conditions. Several parameters were evaluated, such as cell amplification and harvesting yields, cell viability, and cell identity after culture. We observed that microcarriers shape and functionalization impact several A-MSC amplification process steps. However, GhaterDisc-3 microcarriers were the best alternative since harvesting is highly facilitated compared to other microcarriers with a yield of virtually 100 %. Furthermore, A-MSC identity was maintained, since A-MSC keep their characteristic immunophenotype and tri-lineage differentiation ability. Moreover, as these microcarriers are free from any animal derived components, they might be suitable for a use in a GMP compliant large-scale A-MSC production for biomedical applications.

1. Introduction

Human mesenchymal stromal cells (MSC) are self-renewal multipotent cells that can differentiate into a variety of tissues [1]. MSC can be isolated from different tissues sources such as bone marrow, adipose tissue, dental tissues, dermal tissues or umbilical cord [2–4]. Thanks to their facilitated sampling process, adipose tissue derived mesenchymal stromal cells (A-MSC) appear as a really promising source for cell therapy and have already been used in numerous clinical studies [5]. Moreover, it has been suggested that their proliferation capacity is not dependent of the age of the patient [6]. Finally, when compared to bone marrow-derived stem cells, A-MSC seems to be genetically and morphologically more stable, and present a higher proliferation capacity and lower senescence during *in vitro* expansion [7]. Since 2010, the number of clinical trials using A-MSC had steadily increased with some clinical trials in phase III [8]. A-MSC are tested for the treatment of different pathologies such as type 1 diabetes, osteoarthritis, Crohn's disease or heart diseases [9–12]. Even if the immune privileged status of MSC is still under debate [13], several studies highlighted the immunosuppressive properties of A-MSC [14–16], indicating that they could be used for allogeneic graft. This could be a great advantage, since it would allow to prepare stocks of A-MSC from a single donor that could be ready for use when needed. Indeed, according to the treated pathology, as well as the patient, the needed cell dose may be quite consequent, up to hundred of millions per kg body weight of patient [17]. Even in the case of adipose tissue, a good source of A-MSC with a yield of 10^6 cells per 200 mL of fat, A-MSC still need to be amplified up to 10^2 times to obtain a reliable amount of therapeutic cells [18]. Actually, 2D amplification in culture flasks is not adapted, even when using multilayered systems. Indeed, it would be necessary to use approximately 21 flasks (75 cm^2) or three 10-layer flasks to treat one patient (70 kg) with a dose of 2×10^6 cells/kg (1.4×10^8 cells) [19]. Those technics appear cumbersome and cost-effective. Furthermore, they may induce heterogeneity in the final product since cells proliferate in different flasks and are subsequently pooled. Moreover, the important handling steps increase contamination risks. Several studies have shown that the

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phenotype of MSC was dependent of cell expansion process and that continuous passaging could lead to cell transformation [20–22]. To overcome these issues, large scale amplification process in closed-system involving bioreactors have been developed [23–25]. For the culture of anchorage dependent cells such as MSC, especially in the commonly used helix bioreactors or wave bioreactors, microcarriers are needed [26]. Numerous types of microcarriers are commercially available and present different physical properties. They can be solid or porous and made of different materials (dextran, gelatin, polystyrene, cellulose). Microcarriers can also have different shapes (spherical, disc, lens-shape, cylindrical) and surface coatings (extracellular matrix proteins, growth factors, positive charges) [27]. Mechanical properties of the scaffolds on which MSC are amplified have also been widely studied and it was showed that substrate topography and stiffness impact cell fate and alters characteristic MSC immunophenotype [28–34]. Finally, as MSC will be directly injected to patients, it is important to consider microcarriers that are free from any animal components.

In order to develop new microcarriers allowing reproducible *ex-vivo* expansion of clinically grade MSC, all aspects mentioned above and affecting MSC must be considered. Here, we propose a comparative study of different types of microcarriers according to the material used for their synthesis, their shapes and their functionalization. Cytodex-1 and Enhanced attachment are two spherical microcarriers which have already been studied in numerous MSC expansion studies [35–39]. We also tested GhaterDisc-1, GhaterDisc-2 and Ghaterdisc-3 microcarriers developed by the Carroucell company and which were recently patented (Patent FR2000158). The main feature of those microcarriers is their disc flat shape. Indeed, it has been shown that the curvature of spherical microcarriers decreases MSC proliferation because cells are exposed directly to surrounding flows and exposed to high shear stress in stirred bioreactors [40]. Moreover, this disc shape allows a significant increase of the specific surface area of the microcarriers compared to spherical microcarriers without increasing the volume by adjusting the thickness of the disks [41]. This property could permit to increase the amount of cells per unit volume of culture medium. To our knowledge, Carroucell microcarriers are the only disc-shaped microcarriers adapted to stirred culture conditions and not exclusively dedicated for packed-bed bioreactors processes, as it is the case for Fibra-cel® disc microcarriers [42–45].

In this work, we also selected microcarriers according to their functionalization. Enhanced attachment and GhaterDisc-2 microcarriers present hydrophilic components (CellBIND®, an oxygen-containing component on Enhanced attachment microcarriers and Epoxide, a hydrophilic function containing an oxygen atom attached to two adjacent carbon atoms on GhaterDisc-2 microcarriers). Indeed, hydrophilic materials are known to improve cell adhesion due to the adsorption of proteins [46]. We also compared positively charged microcarriers (DEAE on Cytodex-1 and a charged primary amine on Ghaterdisc-3 microcarriers) since positive charges are known to promote cell adhesion due to the adsorption of proteins [46–48]. Finally, we studied the impact of type I Collagen functionalization (GhaterDisc-1 microcarriers), an extracellular matrix component which is widely used to improve cell adhesion and growth on microcarriers [27].

The ability of these five different microcarriers to support A-MSC amplification in static and in dynamic conditions using spinner flasks was compared to the growth of A-MSC in 2D monolayer conditions. We analyzed cell proliferation, cell viability and A-MSC phenotype (expression of A-MSC characteristic immunophenotype, multilineage differentiation potential) after cell expansion with microcarriers. Finally, we determined the yield of cell harvesting, a crucial step in anchorage dependent cells amplification processes.

2. Material and methods

2.1. Cell culture

Adipose tissue derived mesenchymal stromal cells (A-MSC) were provided by the Etablissement Français du Sang (Saint-Ismier, France). Cells were isolated from healthy donors of fat tissue and prepared as previously described. [49] Briefly, human A-MSC were isolated after enzymatic digestion of adipose tissue obtained from subcutaneous abdominal fat of healthy donors during liposuction procedure. Cell expansion of the stromal vascular fraction was performed using the procedures implemented for clinical applications (EFS, France). In order to reduce as much as possible animal derived components within our culture processes, A-MSC (Passage 2–6) isolated from three different donors were cultured in Promocell Mesenchymal stem cell growth medium 2 (Heidelberg, Germany) containing only 2% fetal bovine serum (FBS) at 37 °C and 5% CO2. For cell maintenance and 2D control conditions, cells were seeded on a coating of 10 µg/mL of Human Plasma Fibronectin (FN) from EMD Millipore (Darmstadt, Germany). When cells reached confluence, they were harvested with TrypLE (ThermoFisher, Courtaboeuf, France) and replated into new flasks.

2.2. Microcarriers

Cytodex-1 microcarriers were purchased from GE Healthcare (Buc, France) and Enhanced attachment microcarriers from Corning (Avon, France). GhaterDisc-1, GhaterDisc-2 and Ghaterdisc-3 disc microcarriers were purchased from Carroucell company (Saint Ismier, France). All microcarriers were used according to manufacturer's instructions.

2.3. Cell adhesion assay

96 well plates were covered by different microcarriers or with 10 µg/mL FN. A-MSC were plated at 3×10^4 cells per well for 5, 30, 60, 120 and 180 min. At the end of the kinetic, medium containing unbound cells was slightly removed and replaced by fresh medium containing 1X PrestoBlue reagent (ThermoFisher). Cells were incubated for 2 h at 37 °C, 5% CO_2 and the medium was further transferred into 96 well plates for 590 nm fluorescence measurement. Relative adhered cell amount was determined by comparison between obtained fluorescence values to the one obtained after 180 min adhesion time, since no cells were observed in the removed medium at this time.

For cell visualization on the different microcarriers, A-MSC were prestained with PKH26 red membrane stainer (Sigma-Aldrich) according to the manufacturer instructions. Then, 2×10^4 cells were plated in non-treated 24 well plates, covered by 2 cm^2 of different microcarriers and cultured for 72 h in 400 µL of culture medium. Image acquisition was performed using live microscopy (AxioObserver Z1, Carl Zeiss, Oberkochen, Germany).

2.4. Static culture studies

Static cultures of A-MSC were performed by plating 2×10^4 cells in 400 µL of medium in non-treated 24 well plates, covered by 2 cm^2 of different microcarriers.

For growth kinetics, cells were maintained up to 7 days. Every 3 days, 100 µL of fresh medium was added. At 24, 72, 120 and 168 h, culture medium was removed and 400 µL of fresh medium containing 1X PrestoBlue reagent was added. Fluorescence was then analyzed and compared to growth curves in order to determine cell amount.

For cell death visualization and quantification, A-MSC cells were cultured for 7 days. On day 6, growth medium was removed, and fresh medium containing 1/1000 IncuCyte Cytotox Green reagent (Essen Biosciences, Welwyn Garden City, United Kingdom) was added. On day 7, cells were observed by live imaging for dead cells visualization and death related fluorescence was quantified.

Table 1
Characteristics of microcarriers used in this study.

Microcarrier Characteristics	Enhanced attachment	Cytodex-1	GhaterDisc microcarriers		
Manufacturer	Corning[a]	GE Healthcare[a]	Carroucell[a]		
Material	Polystyrene	Dextran	Silica and 5% Hydroxyapatite (HAP)		
Functionalization	CellBIND	DEAE	Collagen (GhaterDisc-1)	Epoxide (GhaterDisc-2)	NH3 (GhaterDisc-3)
Shape	Spherical beads	Spherical beads	Discs		
Diameter (μm)	168.5 ± 43.5	197.5 ± 50.5	519.5 ± 48.5		
Specific surface ($cm^2\ g^{-1}$)	360	3400 (dry particles)	2500		
Animal components	None	None	2% Collagen (marine origin)	None	

[a] Features are given based on manufacturer data.

2.5. Cell harvesting evaluation

A-MSC were cultured for 96 h. Medium was then removed and cells were rinsed once with Dulbecco's Phosphate Buffer Saline without Ca^{2+}/Mg^{2+} (ThermoFisher). Cells were then incubated with 300 μL of TrypLE dissociation reagent (ThermoFisher). During the cell detachment step, microcarriers settled much faster than detached cells. So, after 10 min, 300 μL of medium was added and microcarrier-free cells were recovered and centrifugated at 1200 rpm for 5 min. The pellet was resuspended in 400 μL of 1X PrestoBlue reagent and the obtained fluorescence amount was compared to the one obtained before cell detachment to determine harvesting yield.

2.6. Dynamic cell culture in spinner flasks

For dynamic cell culture, Techne 4 places Stirrer and Techne 125 mL Spinner Flasks (Techne, Stone, Staffordshire, United Kingdom) were used. A culture volume of 50 mL and a microcarrier surface of 200 cm^2 were selected and 1.5 million cells were seeded. During the first 2 h, stirring was stopped to permit cell attachment. Cells were then cultured for 5 days and the medium was manually refreshed at day 3. At the end of the culture, cells were detached using TryPLE dissociation reagent and separated from the microcarriers using a 100 μm cell strainers (Corning, Wiesbaden, Germany).

2.7. Flow cytometry

Harvested A-MSCs were assessed for surface immunophenotype via

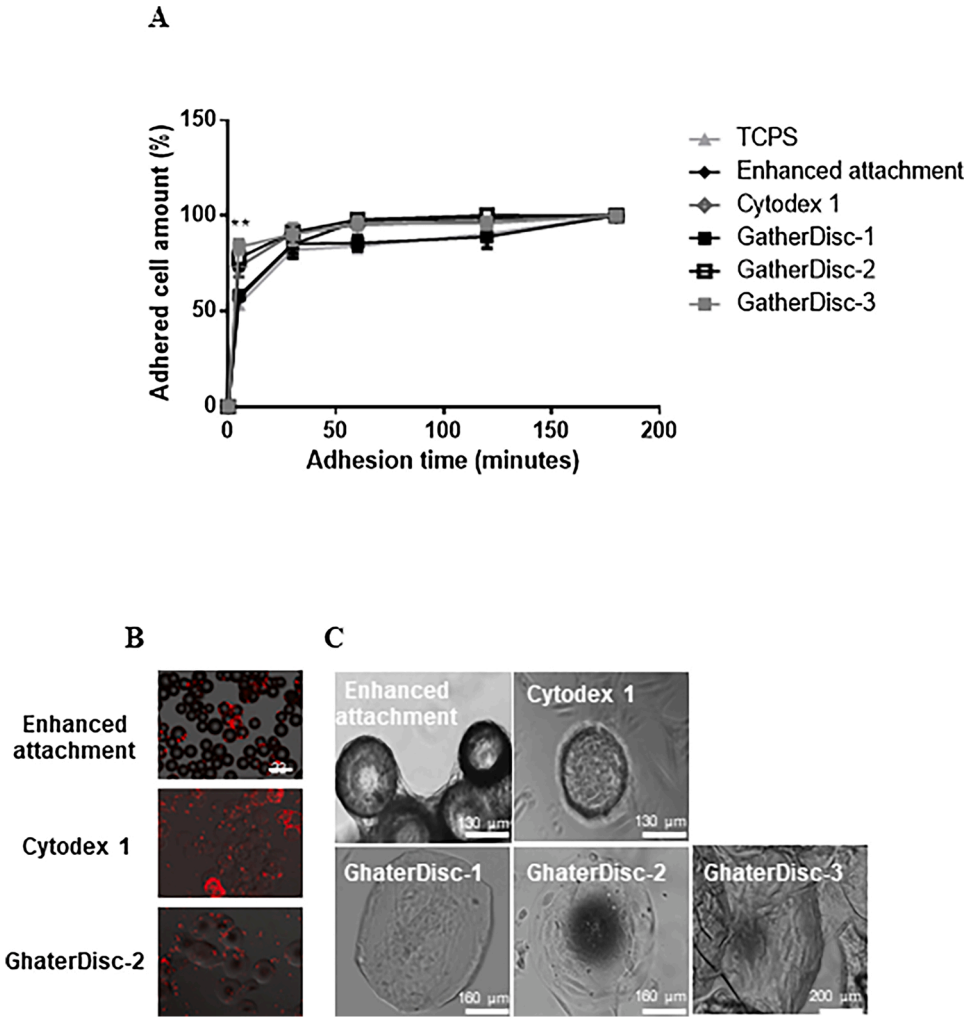

Fig. 1. A) A-MSC adhesion in static conditions according to the microcarriers. At each time, adhesion efficiency was expressed as a fraction of adhered cells compared to maximum number of adhered cells at the end of the kinetic (** indicates $p \leq 0.0016$; $n = 3$). B) Distribution of A-MSC on microcarriers. A-MSC were PKH-26 labeled (red), incubated with microcarriers on static conditions and representative images were taken after 72 h. Images from GhaterDisc-1 and GatherDisc-3 microcarriers could not be acquired because of high autofluorescence giving background signal. C) Phase contrast images of A-MSC attached to the different microcarriers in static conditions after 72 h of expansion.

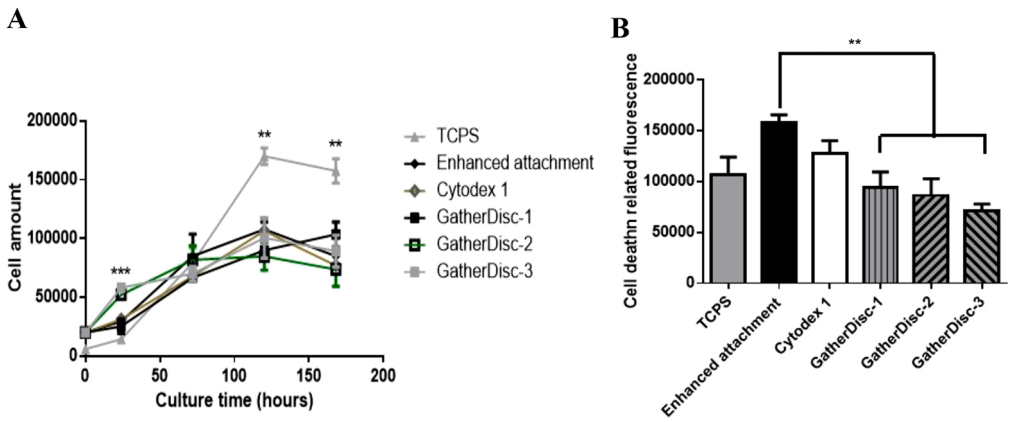

Fig. 2. A-MSC expansion on different types of microcarriers in static conditions. A) Cell growth was monitored over 7 days by using Prestoblue labeling (*** indicates $p \leq 0.0001$; ** indicates $p \leq 0.0032$; $n = 3$). B) Dead cells were evaluated on day 7 of culture by using Cytotox Green reagent. Cell death was relative to the green fluorescence (** indicates $p \leq 0.0045$; $n = 3$).

flow cytometry. Labeling was done with the BD Stemflow Human MSC Analysis Kit (BD Biosciences, Le Pont de Claix, France) according to the manufacturer instructions. Positive markers CD90, CD105 and CD73 where marked with specific antibodies, coupled to FITC, PerCP-Cy5.5 tandem and APC respectively. Negative markers CD45, CD34, CD11b, CD19, HLA-DR were marked with a cocktail of antibodies all coupled to PE. For each sample, 1×10^4 cells were acquired on an Accuri C6 cytometer (BD Biosciences) and data were analyzed with the FCS Express 6 software (De Novo Software, Glendale, USA).

2.8. Tri-lineage differentiation of A-MSC

For adipogenic and osteogenic differentiation, 5×10^4 cells were seeded on 2 wells LAB-TEK II chambers (ThermoFisher). Once 80–90 % confluence was observed, growth media was replaced by AdipoMAX differenciation medium (Sigma-Aldrich) or by Stempro Osteogenesis Differentiation medium (ThermoFisher). For adipogenic differentiation, cells were cultured for 14 days, which corresponds to the optimal time for adipogenic differentiation [50]. The medium was replaced every 4 days. At the end of the culture, cells were washed by PBS and fixed with 4% paraformaldehyde (PFA) for 30 min at room temperature. Cells were rinsed twice with PBS and then stained by Oil Red O (Sigma-Aldrich), during 50 min at room temperature. Slides were washed three times with PBS and mounted for microscope observation. For osteogenic differentiation experiments, cells were cultured for 21 days in differentiation medium that was replaced every 4 days according to published protocols [51]. At the end of the culture, cells were washed twice by PBS and fixed for 20 min with 4% PFA. Cells were washed with distilled water and stained with 2% Alizarin Red, pH 4.2 for 20 min at room temperature. Unspecific staining was removed with distilled water and slide was mounted for microscope observation. To determine the chondrogenesis potential of A-MSC, 1×10^5 cells were seeded on 96-well Spheroid Microplates (Corning). After 1 or 2 days, when the spheroids formed, growth medium was replaced by Stempro Chondrogenesis Differentiation medium (ThermoFisher). Cells were cultured for 21 days, a sufficient time to observe chondrogenesis differentiation [51]. The medium was changed every 4 days. At the end of the culture, spheroids were pooled, washed once with PBS and fixed with 4% PFA overnight at 4 °C. They were washed twice with PBS and embedded in Tissue-Tek O.C.T compound (Gentaur, Paris, France) for cryosection. 10 µm sections were then cut and transferred on Superfrost slides. Cryosections were then stained with 1% Alcian Blue (Sigma-Aldrich) for 30 min, protected from light. Unspecific staining was removed by three washes with HCL 0.1 N and slides were mounted for microscope observation.

2.9. Cell senescence study

A-MSC were plated on 2 wells LAB-TEK II chambers (1×10^4 cells per well). After 3–4 days, cells were stained with the Senescence β-Galactosidase Staining Kit (Cell Signaling, Leiden, The Netherlands), according to the manufacturer instructions. Slides were mounted for microscope observation and 10 pictures were randomly taken for each condition. β-Galactosidase positive cells were then counted and normalized to the total number of cells in each field.

2.10. Statistical analysis

Data were expressed as the mean value ± the standard deviation (mean ± SD) and analyzed by a two-tailed unpaired *t*-test. The degree of statistical significance was set at $P < 0.05$.

3. Results

3.1. A-MSC adhesion and proliferation on microcarriers in static conditions

In a recent study, Rafiq et al. highlighted the reliability of a static systematic screening to predict the efficiency of microcarriers for MSC amplification in stirred environment [52]. Therefore, the first part of our study was realized in static conditions. The impact of microcarriers properties (Table 1) on cell adhesion, proliferation, mortality, and harvesting was evaluated.

The efficiency of cell adhesion at the beginning of culture would influence cell expansion, since a latency would occur if a too low number of cells adhered on microcarriers. A-MSC attachment on microcarriers was compared to that observed on Tissue Culture Polystyrene plates (TCPS), used as control surfaces. After 30 min on static conditions, about 80 % of cells adhered on all microcarriers (Fig. 1A). For shorter times, we could observe that GhaterDisc-2, Ghaterdisc-3 as well as Cytodex-1 microcarriers promoted a significant increase of A-MSC adhesion. More than 75 % of cells were attached to these microcarriers after 5 min. This was much faster than adhesion on Enhanced-attachment or GhaterDisc-1 microcarriers and on TCPS plates.

One crucial aspect in microcarriers dependent cell culture in bioreactors is to allow a uniform cell distribution during the attachment phase at the beginning of the process [53]. Classically, a static attachment phase is used and we assumed that microcarriers properties might play a key role in the cell distribution on them. Cell distribution on microcarriers was observed after 72 h of incubation without agitation. A-MSC are heterogeneously distributed on Enhanced-attachment and Cytodex-1 microcarriers, with some microcarriers without cells and

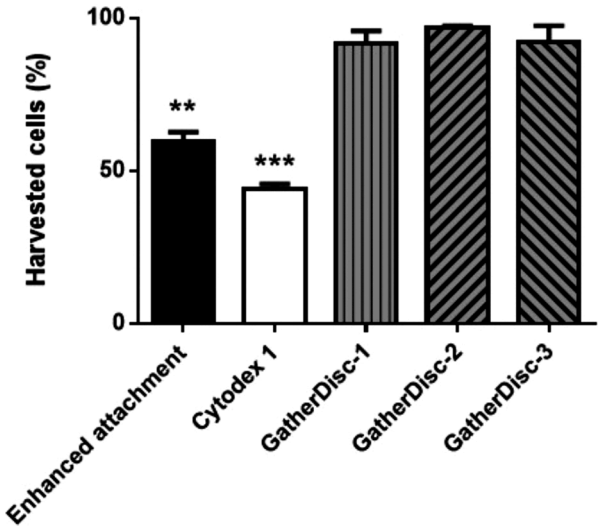

Fig. 3. A-MSC harvesting from the different microcarriers studied. The fraction of detached cells is represented according to the total number of cells in each condition (** indicates $p \leq 0.0064$ and *** indicates $p \leq 0.001$; $n = 3$).

others with a high cell density (Fig. 1B). This leads to cell aggregates that may not be desirable, since high cell–cell contact may negatively affect the proliferation and differentiation potential of A-MSC. [54] Conversely, A-MSC appeared more homogeneous distributed on GatherDisc microcarriers since all microcarriers seemed to be colonized. Concerning cells morphology, A-MSC were notably more spread with a fibroblastic shape on GatherDisc microcarriers than on Enhanced-attachment or Cytodex-1 microcarriers (Fig. 1C), indicating that disc flat form allowed to keep A-MSC phenotype during expansion microcarriers.

As shown on Fig. 2A, A-MSC proliferated on all microcarriers, but with slight differences. After one day, Ghaterdisc-3 and GhaterDisc-2 microcarriers showed the best ability to promote cell growth ($p \leq 0.0001$). However, after 5 days, we could obtain the same number of cells with all microcarriers. It should be noted that for all tested microcarriers, the total number of A-MSC remained approximately 1.5 times less than that obtained on TCPS plates after 7 days. Finally, at the end of the culture, the proliferation seemed to decline except with GhaterDisc-1 microcarriers. This could reflect cell death or a lack of free surface on microcarriers. Indeed, after 7 days on microcarriers, the cell death on Enhanced-attachment and Cytodex-1 microcarriers was significantly increased by 1.5 and 1.2 times respectively compared to that observed on TCPS plates (Fig. 2B). On the contrary, cell death was reduced by a factor of 1.1, 1.25 and 1.7 with GhaterDisc-1, GhaterDisc-2 and Ghaterdisc-3 microcarriers respectively. The elevated cell death observed with Enhanced-attachment and Cytodex-1 microcarriers could reflect the high number of aggregates observed with these class of microcarriers (Fig. 1B, Fig. S1).

So, in static conditions, GatherDisc microcarriers appeared particularly adapted for the amplification of A-MSC, since for a same microcarriers available surface (2 cm^2), they promoted A-MSC adhesion and proliferation with the same rate as other available microcarriers currently used, while allowing a better cell survival, which is a crucial criterion for the amplification of therapeutic cells.

3.2. A-MSC harvesting from microcarriers in static conditions

The ease of detachment of cells from microcarriers is a major challenge in microcarriers-based cell culture. Based on an analysis of the literature, we decided to test TrypLE, a xenofree recombinant trypsin. [55] In the same conditions of enzyme concentration and time of incubation, we could observe that virtually 100 % of the cells have been recovered from GatherDisc microcarriers, whatever the functionalization (Fig. 3). On the contrary, half of the cells remained attached to Enhanced-attachment or Cytodex-1 microcarriers. Therefore, GhaterDisc-1, GhaterDisc-2 and Ghaterdisc-3 microcarriers offer a valuable benefit compared to other microcarriers concerning the harvesting step.

3.3. A-MSC expansion under stirring conditions in spinner flasks

To further characterize the different tested microcarriers, we studied their performance in spinner flasks. For each assay, 1.5×10^6 A-MSC were inoculated in low serum medium containing an identical total microcarriers surface of 200 cm^2. After 5 days, we observed that each microcarrier type could promote A-MSC proliferation in stirred conditions (Fig. 4A). However, GhaterDisc-2 microcarriers seemed to be the less suitable for A-MSC amplification in these conditions. On the contrary, Enhanced-attachment, Cytodex 1, GhaterDisc-1 and GatherDisc-3 microcarriers were able to generate significantly higher cell expansion ability (6.3–8 fold expansion) of A-MSC compared to GatherDisc-2 microcarriers. In contrast, we observed an important difference in cell viability among the more efficient microcarriers. For example, cells harvested from Ghaterdisc-3 and Cytodex 1 microcarriers presented a significantly higher cell viability of 95.3 ± 1.15 % and 96 ± 1.4 % respectively compared to 90 ± 2% with Enhanced-attachment microcarriers (Fig. 4B). This difference was previously observed in static conditions (Fig. 2). It is of particular importance since it is necessary to develop process minimizing adverse effects on the cells to obtain healthy A-MSC for a clinical use.

Finally, senescence was quantified, since it indicates a progressive loss of proliferative potential that is associated with a reduction of A-MSC therapeutic properties [56]. Regarding senescence rate, it appeared significantly less important with Enhanced-attachment and GhaterDisc-1 microcarriers (Fig. 4C). Senescent cells represent about 14 % of total cells, which is of the same order as what we could observed with A-MSC growing on TCPS plates (data not shown). On the contrary, about one fifth of the cells appeared senescent with Cytodex-1 and GhaterDisc-2 microcarriers. Finally, senescence rate is at an intermediate level of 16.2 ± 4.2 % with GatherDisc-3 microcarriers. Interestingly, same tendencies in term of cell yields, cell viability and cell senescence were obtained after the growth of A-MSC originated from three different donors on Enhanced attachment, Cytodex 1 and GatherDisc-1 microcarriers (Table S1, Supplemental Information). These results emphasize the robustness of the culture conditions that we applied in our study.

3.4. A-MSC identity validation after amplification on microcarriers

Finally, it was important to determine whether the A-MSC harvested after amplification on microcarriers retained their phenotype and differentiation capacity. Cells were detached from microcarriers after 5 days of growth in spinner flasks and analyzed by flow cytometry for three positive markers (CD73, CD90 and CD105) and five negative markers (CD11b, CD19, CD34, CD45, HLA-DR). For almost all tested microcarriers, the cell expression was >95 % for the positive markers (Fig. 5A) and <2% for the negative markers (Table S3, Supporting Information). Notably, a significant decrease of CD90 and CD105 expression level was observed on A-MSC grown on GatherDisc-1 (Fig. 5A, Table S2). The ability of harvested cells to differentiate into adipocytes, osteocytes and chondrocytes was also analyzed by Oil Red, Alizarin red and Alcian Blue staining respectively (Fig. 5B). Our results demonstrated that the amplification of A-MSC on microcarriers preserved their tri-lineage differentiation potential. All these results confirmed that after expansion in stirred conditions followed by their harvesting, A-MSC grown on Enhanced attachment, Cytodex 1, GatherDisc-2 and GatherDisc-3 microcarriers, and to a lesser extent on GatherDisc-1 microcarriers, kept their mesenchymal stromal cells phenotype according to the ISCT (International Society for Cell and

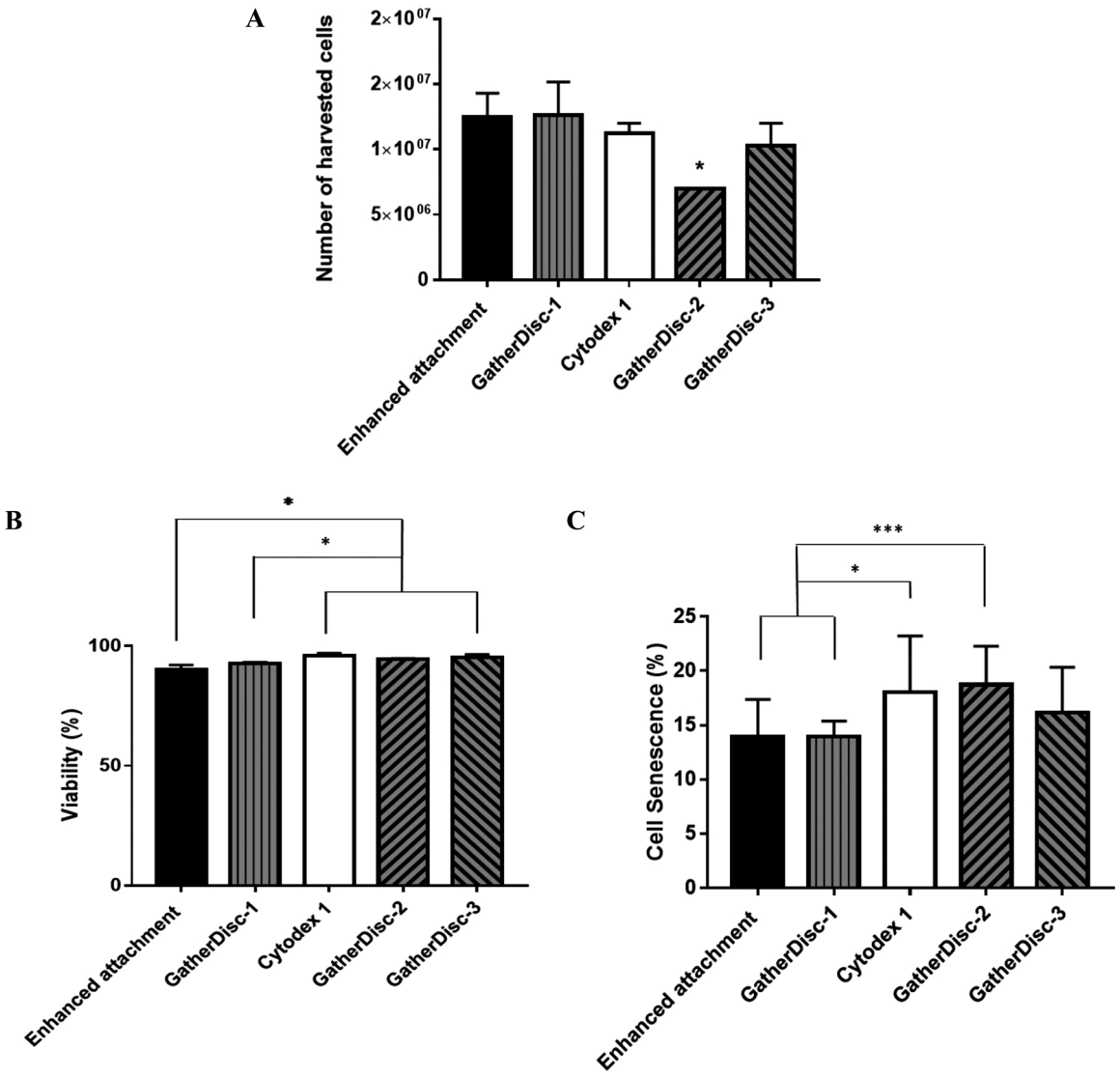

Fig. 4. Cell yield (A), viability (B), and senescence (C) of A-MSC after amplification in spinner flask according to the type of microcarriers (* indicates $p \leq 0{,}0324$; *** indicates $p \leq 0{,}0006$; $n = 3$).

Gene Therapy) [57,58].

4. Discussion

The effective transfer of human MSC into clinical practice will depend on the development of scalable and cost-effective processes for their amplification. Microcarriers offer a convenient method for growing adherent cells because of their high surface/volume ratio and the use of a stirred bioreactor allowing to monitor and to control environmental parameters (temperature, pH, pO2). A tremendous amount of different microcarriers are currently developed by manufacturers and commercialized for anchorage-dependent cells amplifications processes. Systematic screening of several different microcarriers is therefore a key step to select the better microcarriers allowing MSC amplification processes while maintaining their therapeutic properties [39,52,59]. One of the main differences among the tested microcarriers is their shape since both Enhanced attachment and Cytodex-1 microcarriers are spherical, whereas Carroucell microcarriers have an innovative disc shape. This allows to increase the surface without increasing the volume by adjusting the thickness, and then improves the surface/volume ratio, thereby explaining the specific surface of 2500 $cm^2\ g^{-1}$ obtained with those microcarriers (Table 1).

Although no substantial differences were observed on A-MSC adhesion kinetics between microcarriers, whatever the functionalization, a dramatic increase of culture homogeneity was observed on Carroucell microcarriers in comparison with spherical ones (Fig. 1). As shown by Hu et al. cells adhere to microcarriers following a Poisson distribution law in which the probability to have non-colonized microcarriers within the culture is inversely correlated with the number of cells inoculated per microcarriers [60]. Due to their high specific surface, and for a same seeding density (1×10^4 cells per cm^2), the number of A-MSC inoculated per GatherDisc microcarrier (40 cells) is dramatically higher than the one on spherical Enhanced attachment and Cytodex-1 microcarriers (10 cells per microcarrier), which may explain this difference of culture homogeneity. This is of particular importance for expansion of A-MSC since it is necessary to achieve confluence on all microcarriers at the same time to ensure a high-quality cell preparation.

We observed that cell mortality is significantly higher on both Enhanced attachment microcarriers in comparison with microcarriers from Carroucell (Fig. 2). Our hypothesis is that the heterogeneity observed on spherical microcarriers cultures is amplified along the culture time, thereby forming high cell concentration areas and then microcarriers - cells aggregates, known to alter cell viability (Fig. S1, Supporting Information) [53,61].

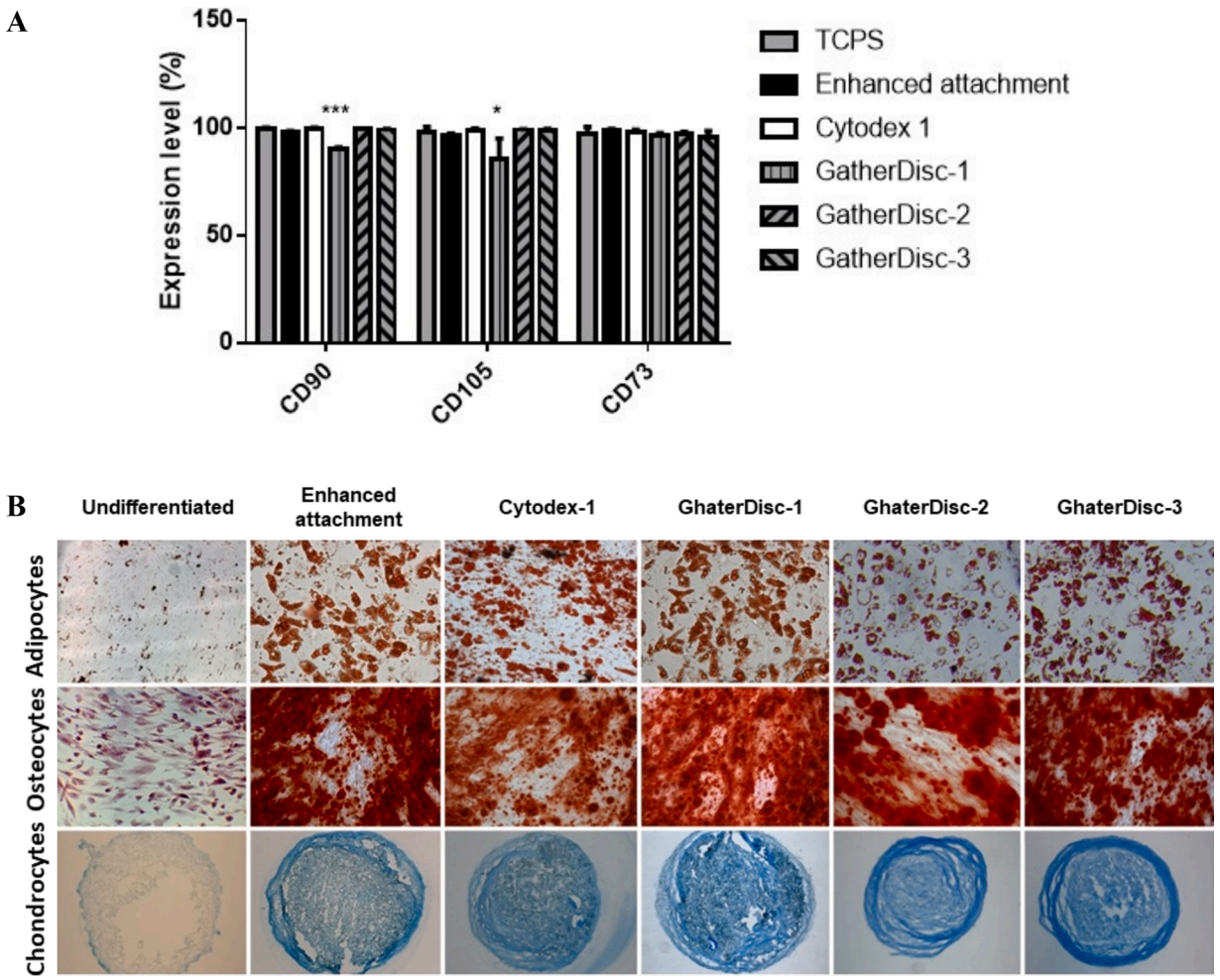

Fig. 5. Characterization of A-MSC (Donor 2) harvested from the different microcarriers after spinner flask amplification. A) Flow cytometry analysis of expression of positive cell surface markers (CD73, CD90, and CD105) (*** indicates $p < 0.0001$; * indicates $p = 0.0437$; $n = 3$). B) *in vitro* differentiation of A-MSC into adipocytes (Oil red staining, top panel), osteocytes (Alizarin red staining, middle panel) or chondrocytes (Alcian blue staining, bottom panel). Negative control (Undifferentiated cells) showed staining of A-MSC in basal medium, without differentiation factors.

Cell harvesting from microcarriers at the end of the culture is a crucial step to obtain important yields of therapeutic MSC. Once again, we observed that the shape of microcarriers rather than functionalization has a considerable impact on A-MSC harvesting efficiency (Fig. 3). Indeed, 60 % and 45 % of A-MSC could be detached from Enhanced attachment and Cytodex-1 spherical microcarriers respectively whereas 100 % of cells could be harvested from the three Carroucell microcarriers, even if they presented different functionnalization. We assume that due to their planar surfaces, disc microcarriers promote dissociation enzyme action since A-MSC morphology is similar with the one observed on TCPS (Fig. 1C).

The next step of our study was to evaluate microcarriers suitability for A-MSC dynamic culture in spinner-flasks. GatherDisc-3 microcarriers appear as the best alternative for the production of healthy A-MSC. Indeed, A-MSC yields were significantly higher than the ones obtained with GatherDisc-2 microcarriers and statistically similar than all the other microcarriers (Fig. 4A). Moreover, cells presented a significantly higher cell viability (Fig. 4B), as well as a moderate cell senescence rate (Fig. 4C). Finally, production of clinical-grade A-MSC should comply with GMP (Good Manufacturing Practice) and avoid the use of animal-derived substances [62]. In this context also, Ghaterdisc-3 microcarriers appeared as the best alternative since their matrix is only functionalized with positively charged chemical functions. Moreover, in comparison with spherical xeno-free microcarriers (Enhanced-attachment and Cytodex 1 microcarriers), a higher harvesting ability is observed (Fig. 3) which might be of major impact for further large scale A-MSC amplification process. Finally, A-MSC kept their characteristic immunophenotype as well as their tri-lineage differentiation ability after the culture on those GatherDisc-3 microcarriers (Fig. 5).

However, these results must be completed by checking out the functionality of A-MSC following their amplification on the different microcarriers. The tri-lineage differentiation ability of cells must be quantitatively evaluated by analyzing the expression of specific markers such as adiponectin for adipogenesis, Sox9 for chondrogenesis or RunX2 for osteogenesis [58]. As bone marrow derived MSC, A-MSC are known for their immunomodulation properties [63]. Then, the functionality of the cells could be analyzed by quantifying the production of immunomodulative factors such as IL-6 or TGF-β1.

It should be noted that the performance of microcarrier-based cell expansion is largely dependent of the agitation system. This one should allow having a homogeneous suspension of microcarriers to guarantee sufficient mass transfer of nutrients and oxygen to the cells. On the other hand, hydrodynamic shear stress generated by the agitation must be reduced to limit damages to the cells [64,65]. In our study, we used the Techne Spinner Flask model characterized by a unique rod shape impeller. We observed that GatherDisc microcarriers tended to sediment after 4–5 days of dynamic culture (data not shown) suggesting that the agitation regimen was not optimized for disc-shape microcarriers. In order to obtain best cell amplification performances, one of our perspective will be to adapt the impeller system to each type of

Table 2
Summary of the results presented in this study.

			Xeno-free microcarriers			
Microcarriers Features*		GhaterDisc-1	Enhanced attachment	Cytodex-1	GhaterDisc-2	Ghaterdisc-3
Yields		+++	+++	+++	+	+++
Viability of A-MSC		++	++	+++	+++	+++
Harvesting of A-MSC		+++	+	+	+++	+++
	-Lack of Senescence	+++	+++	+	+	++
Identity of A-MSC	-Differentiation ability	+++	+++	+++	+++	+++
	-Surface markers expression	+	+++	+++	+++	+++

* Features relates to results obtained after amplification of A-MSC in dynamic conditions (spinner-flasks).

microcarrier according to its properties. Furthermore, wave bioreactors, characterized by really different agitation systems, have already been used for MSC amplification on microcarriers [66–68]. Therefore, the next step of our study would be to highlight the best microcarrier-bioreactor association for A-MSC amplification efficiency.

Herein, A-MSC culture was performed under low fetal bovine-containing medium (2% of FBS). Until now, very few studies have been performed on the amplification of A-MSC with microcarriers in total xeno-free conditions. A major limitation was to promote efficient adhesion of A-MSC on microcarriers in serum-free medium. [37,69] Indeed, serum serve to deliver Extracellular Matrix proteins that are required for the interaction of the cells with the microcarriers. Cunha et al. proposed a xeno-free process for A-MSC amplification using Synthemax microcarriers [70]. They successfully expanded A-MSC in a 2 L bioreactor, but during harvesting, they lost 25 % of the cells. Furthermore, less than 95 % of A-MSC expressed CD105 marker, indicating the impact of their amplification process on cell phenotype. Further studies would be conducted to test the performance of different xeno-free microcarriers in xeno-free media. Some synthetic media have already been developed and are commercially available for xeno-free cell amplification process. Human platelet lysate represent also an alternative to fetal bovine serum [71]. In order to develop a GMP compliant process, it may be interesting to combine xeno-free Ghaterdisc-3 microcarriers, which appeared as the best alternative, with different serum-free media.

Finally, it should be noted that large-scale expansion of stem cells is needed for every types of stem-cells-based therapies. In this context, Chen et al. realized a screening of different microcarriers for the amplification of non-differentiated embryonic stem cells (ES) [72]. Studying both spherical and cylindrical microcarriers, these researchers highlighted the impact of the size and shape of those microcarriers on cell growth and stemness. Similarly, some groups have developed microcarrier-based systems for the amplification of induced pluripotent stem cells (iPS). This is challenging as these cells grow in multilayer colonies on extracellular matrices and are more susceptible to shear stress. Even if there is no consensus regarding which microcarrier was the most suitable iPSC expansion, it appeared that microcarrier size and shape impact cell morphology [73]. Therefore, it could be interesting to compare spherical microcarriers to disc-shape microcarriers from Carroucell for the amplification of other therapeutic stem cells, such as ES, but also iPS.

5. Conclusion

Our study indicated that the shape of the microcarriers, more than the functionalization, had a major impact on A-MSC behavior during the expansion process. In this context, Ghaterdisc-3 disc microcarriers might be a real alternative for A-MSC amplification process, as highlighted in Table 2. Further studies should now be conducted to determine their efficiency for A-MSC amplification in xeno-free medium, as well as in more adapted agitation systems. We would then confirm if those microcarriers could be a new alternative for A-MSC production for a clinical use.

Author contribution statement

Tristan Le Clainche - Data curation; Formal analysis; Investigation; Methodology; Roles/Writing - original draft; Writing - review & editing.

Anaïck Moisan - Methodology; Review & editing.

Jean-Luc Coll – Conceptualization; Project administration; Resources; Supervision; Review & editing.

Véronique Martel-Frachet - Conceptualization; Formal analysis; Investigation; Methodology; Project administration; Resources; Supervision; Roles/Writing - original draft; Writing - review & editing.

Declaration of Competing Interest

The authors report no declarations of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.bej.2021.108082.

References

[1] X. Wei, X. Yang, Z. Han, F. Qu, L. Shao, Y. Shi, Mesenchymal stem cells: a new trend for cell therapy, Acta Pharmacol. Sin. 34 (2013) 747–754, https://doi.org/10.1038/aps.2013.50.

[2] G. Bartsch, J.J. Yoo, P. De Coppi, M.M. Siddiqui, G. Schuch, H.G. Pohl, J. Fuhr, L. Perin, S. Soker, A. Atala, Propagation, expansion, and multilineage differentiation of human somatic stem cells from dermal progenitors, Stem Cells Dev. 14 (2005) 337–348, https://doi.org/10.1089/scd.2005.14.337.

[3] G.T.-J. Huang, S. Gronthos, S. Shi, Mesenchymal stem cells derived from dental tissues vs. those from other sources: their biology and role in regenerative medicine, J. Dent. Res. 88 (2009) 792–806, https://doi.org/10.1177/0022034509340867.

[4] S. Kern, H. Eichler, J. Stoeve, H. Klüter, K. Bieback, Comparative analysis of mesenchymal stem cells from bone marrow, umbilical cord blood, or adipose tissue, Stem Cells 24 (2006) 1294–1301, https://doi.org/10.1634/stemcells.2005-0342.

[5] A.S. Şovrea, A.B. Boşca, A.M. Constantin, E. Dronca, A. Ilea, State of the art in human adipose stem cells and their role in therapy, Rom. J. Morphol. Embryol. 60 (2019) 7–31.

[6] O.S. Beane, V.C. Fonseca, L.L. Cooper, G. Koren, E.M. Darling, Impact of aging on the regenerative properties of bone marrow-, muscle-, and adipose-derived mesenchymal stem/stromal cells, PLoS One 9 (2014), https://doi.org/10.1371/journal.pone.0115963 e115963.

[7] L. Frese, P.E. Dijkman, S.P. Hoerstrup, Adipose tissue-derived stem cells in regenerative medicine, Transfus. Med. Hemother. 43 (2016) 268–274, https://doi.org/10.1159/000448180.

[8] P. Van Pham, Adipose stem cells in the clinic, Biomed. Res. Ther. 1 (2014) 11, https://doi.org/10.7603/s40730-014-0011-8.

[9] Y.B. Cho, K.J. Park, S.N. Yoon, K.H. Song, D.S. Kim, S.H. Jung, M. Kim, H.Y. Jeong, C.S. Yu, Long-term results of adipose-derived stem cell therapy for the treatment of Crohn's fistula: ASCs for the treatment of Crohn's fistula, Stem Cells Transl. Med. 4 (2015) 532–537, https://doi.org/10.5966/sctm.2014-0199.

[10] H.-P. Lin, T.-M. Chan, R.-H. Fu, C.-P. Chuu, S.-C. Chiu, Y.-H. Tseng, S.-P. Liu, K.-C. Lai, M.-C. Shih, Z.-S. Lin, H.-S. Chen, D.-C. Yeh, S.-Z. Lin, Applicability of adipose-derived stem cells in type 1 diabetes mellitus, Cell Transplant. 24 (2015) 521–532, https://doi.org/10.3727/096368915X686977.

[11] E.C. Perin, R. Sanz-Ruiz, P.L. Sánchez, J. Lasso, R. Pérez-Cano, J.C. Alonso-Farto, E. Pérez-David, M.E. Fernández-Santos, P.W. Serruys, H.J. Duckers, J. Kastrup, S. Chamuleau, Y. Zheng, G.V. Silva, J.T. Willerson, F. Fernández-Avilés, Adipose-derived regenerative cells in patients with ischemic cardiomyopathy: the PRECISE trial, Am. Heart J. 168 (2014) 88–95, https://doi.org/10.1016/j.ahj.2014.03.022, e2.

[12] Y.-M. Pers, C. Jorgensen, Adipose derived stem cells for regenerative therapy in osteoarticular diseases, Horm. Mol. Biol. Clin. Investig. 28 (2016), https://doi.org/10.1515/hmbci-2016-0010.

[13] J.A. Ankrum, J.F. Ong, J.M. Karp, Mesenchymal stem cells: immune evasive, not immune privileged, Nat. Biotechnol. 32 (2014) 252–260, https://doi.org/10.1038/nbt.2816.

[14] Y. Seo, T.-H. Shin, H.-S. Kim, Current strategies to enhance adipose stem cell function: an update, IJMS 20 (2019) 3827, https://doi.org/10.3390/ijms20153827.

[15] L. Mazini, L. Rochette, M. Amine, G. Malka, Regenerative capacity of adipose derived stem cells (ADSCs), comparison with mesenchymal stem cells (MSCs), IJMS 20 (2019) 2523, https://doi.org/10.3390/ijms20102523.

[16] B. Puissant, C. Barreau, P. Bourin, C. Clavel, J. Corre, C. Bousquet, C. Taureau, B. Cousin, M. Abbal, P. Laharrague, L. Penicaud, L. Casteilla, A. Blancher, Immunomodulatory effect of human adipose tissue-derived adult stem cells: comparison with bone marrow mesenchymal stem cells, Br. J. Haematol. 129 (2005) 118–129, https://doi.org/10.1111/j.1365-2141.2005.05409.x.

[17] A. Mizukami, K. Swiech, Mesenchymal stromal cells: from discovery to manufacturing and commercialization, Stem Cells Int. 2018 (2018), https://doi.org/10.1155/2018/4083921.

[18] M. Witkowska-Zimny, K. Walenko, Stem cells from adipose tissue, Cell. Mol. Biol. Lett. 16 (2011), https://doi.org/10.2478/s11658-011-0005-0.

[19] P.A. Tozetti, S.R. Caruso, A. Mizukami, T.R. Fernandes, F.B. da Silva, F. Traina, D. T. Covas, M.D. Orellana, K. Swiech, Expansion strategies for human mesenchymal stromal cells culture under xeno-free conditions, Biotechnol. Prog. 33 (2017) 1358–1367, https://doi.org/10.1002/btpr.2494.

[20] J. Cai, X. Miao, Y. Li, C. Smith, K. Tsang, L. Cheng, Q. Wang, Whole-genome sequencing identifies genetic variances in culture-expanded human mesenchymal stem cells, Stem Cell Rep. 3 (2014) 227–233, https://doi.org/10.1016/j.stemcr.2014.05.019.

[21] S. Neri, P. Bourin, J.-A. Peyrafitte, L. Cattini, A. Facchini, E. Mariani, Human adipose stromal cells (ASC) for the regeneration of injured cartilage display genetic stability after in vitro culture expansion, PLoS One 8 (2013) e77895, https://doi.org/10.1371/journal.pone.0077895.

[22] M. Patrikoski, M. Juntunen, S. Boucher, A. Campbell, M.C. Vemuri, B. Mannerström, S. Miettinen, Development of fully defined xeno-free culture system for the preparation and propagation of cell therapy-compliant human adipose stem cells, Stem Cell Res. Ther. 4 (2013) 27, https://doi.org/10.1186/scrt175.

[23] A. Lakowitz, R. Krull, R. Biedendieck, Recombinant production of the antibody fragment D1.3 scFv with different Bacillus strains, Microb. Cell Fact. 16 (2017) 14, https://doi.org/10.1186/s12934-017-0625-9.

[24] J.R. Price, W.K. Shieh, C.M. Sales, A novel bioreactor for high density cultivation of diverse microbial communities, J. Vis. Exp. (2015) 53443, https://doi.org/10.3791/53443.

[25] F. Tapia, D. Vázquez-Ramírez, Y. Genzel, U. Reichl, Bioreactors for high cell density and continuous multi-stage cultivations: options for process intensification in cell culture-based viral vaccine production, Appl. Microbiol. Biotechnol. 100 (2016) 2121–2132, https://doi.org/10.1007/s00253-015-7267-9.

[26] A.L. van Wezel, Growth of cell-strains and primary cells on micro-carriers in homogeneous culture, Nature 216 (1967) 64–65.

[27] H. Tavassoli, S.N. Alhosseini, A. Tay, P.P.Y. Chan, S.K. Weng Oh, M.E. Warkiani, Large-scale production of stem cells utilizing microcarriers: a biomaterials engineering perspective from academic research to commercialized products, Biomaterials 181 (2018) 333–346, https://doi.org/10.1016/j.biomaterials.2018.07.016.

[28] B.M. Baker, B. Trappmann, W.Y. Wang, M.S. Sakar, I.L. Kim, V.B. Shenoy, J. A. Burdick, C.S. Chen, Cell-mediated fibre recruitment drives extracellular matrix mechanosensing in engineered fibrillar microenvironments, Nat. Mater. 14 (2015) 1262–1268, https://doi.org/10.1038/nmat4444.

[29] N. Huebsch, E. Lippens, K. Lee, M. Mehta, S.T. Koshy, M.C. Darnell, R.M. Desai, C. M. Madl, M. Xu, X. Zhao, O. Chaudhuri, C. Verbeke, W.S. Kim, K. Alim, A. Mammoto, D.E. Ingber, G.N. Duda, D.J. Mooney, Matrix elasticity of void-forming hydrogels controls transplanted-stem-cell-mediated bone formation, Nat. Mater. 14 (2015) 1269–1277, https://doi.org/10.1038/nmat4407.

[30] J.S. Park, J.S. Chu, A.D. Tsou, R. Diop, Z. Tang, A. Wang, S. Li, The effect of matrix stiffness on the differentiation of mesenchymal stem cells in response to TGF-β, Biomaterials 32 (2011) 3921–3930, https://doi.org/10.1016/j.biomaterials.2011.02.019.

[31] J. Xie, M. Bao, S.M.C. Bruekers, W.T.S. Huck, Collagen gels with different fibrillar microarchitectures elicit different cellular responses, ACS Appl. Mater. Interfaces 9 (2017) 19630–19637, https://doi.org/10.1021/acsami.7b03883.

[32] C. Yang, M.W. Tibbitt, L. Basta, K.S. Anseth, Mechanical memory and dosing influence stem cell fate, Nat. Mater. 13 (2014) 645–652, https://doi.org/10.1038/nmat3889.

[33] B. Zhang, N. Kasoju, Q. Li, J. Ma, A. Yang, Z. Cui, H. Wang, H. Ye, Effect of substrate topography and chemistry on human mesenchymal stem cell markers: a transcriptome study, IJSC 12 (2019) 84–94, https://doi.org/10.15283/ijsc18102.

[34] T.L. Clainche, D. Linklater, S. Wong, P. Le, S. Juodkazis, X.L. Guével, J.-L. Coll, E. P. Ivanova, V. Martel-Frachet, Mechano-bactericidal titanium surfaces for bone tissue engineering, ACS Appl. Mater. Interfaces 12 (2020) 48272–48283, https://doi.org/10.1021/acsami.0c11502.

[35] S. Frauenschuh, E. Reichmann, Y. Ibold, P.M. Goetz, M. Sittinger, J. Ringe, A microcarrier-based cultivation system for expansion of primary mesenchymal stem cells, Biotechnol. Prog. 23 (1) (2007) 187–193, https://doi.org/10.1021/bp060155w.

[36] J. Hupfeld, I.H. Gorr, C. Schwald, N. Beaucamp, K. Wiechmann, K. Kuentzer, R. Huss, B. Rieger, M. Neubauer, H. Wegmeyer, Modulation of mesenchymal stromal cell characteristics by microcarrier culture in bioreactors: cultivation-dependent MSC modulation, Biotechnol. Bioeng. 111 (2014) 2290–2302, https://doi.org/10.1002/bit.25281.

[37] J.G. Carmelo, A. Fernandes-Platzgummer, M.M. Diogo, C.L. da Silva, J.M.S. Cabral, A xeno-free microcarrier-based stirred culture system for the scalable expansion of human mesenchymal stem/stromal cells isolated from bone marrow and adipose tissue, Biotechnol. J. 10 (2015) 1235–1247, https://doi.org/10.1002/biot.201400586.

[38] A.C. Tsai, T. Ma, Expansion of human mesenchymal stem cells in a microcarrier bioreactor, Methods Mol. Biol. 1502 (2016) 27032950, https://doi.org/10.1007/7651_2016_338.

[39] D. Schop, R. van Dijkhuizen-Radersma, E. Borgart, F.W. Janssen, H. Rozemuller, H.-J. Prins, J.D. de Bruijn, Expansion of human mesenchymal stromal cells on microcarriers: growth and metabolism, J. Tissue Eng. Regen. Med. 4 (2010) 131–140, https://doi.org/10.1002/term.224.

[40] J.J. Schmidt, J. Jeong, H. Kong, The interplay between cell adhesion cues and curvature of cell adherent alginate microgels in multipotent stem cell culture, Tissue Eng. Part A 17 (2011) 2687–2694, https://doi.org/10.1089/ten.tea.2010.0685.

[41] P.A. Webb, C. Orr, Analytical Methods in Fine Particle Technology, 1. ed., 2. print, Micromeritics, Norcross, 1997.

[42] Y.C. Hu, J.T. Lu, Y.C. Chung, High-density cultivation of insect cells and production of recombinant baculovirus using a novel oscillating bioreactor, Cytotechnology (2003), https://doi.org/10.1023/b:cyto.0000015841.98225.27.

[43] W. Liu, D. Hu, C. Gu, Y. Zhou, W.S. Tan, Fabrication and in vitro evaluation of a packed-bed bioreactor based on an optimum two-stage culture strategy, J. Biosci. Bioeng. 127 (4) (2019) 506–514, https://doi.org/10.1016/j.jbiosc.2018.09.010.

[44] A. McCarron, M. Donnelley, C. McIntyre, D. Parsons, Transient lentiviral vector production using a packed-bed bioreactor system, Hum. Gene Ther. Methods (2019), https://doi.org/10.1089/hgtb.2019.038.

[45] G. Wang, W. Zhang, C. Jacklin, D. Freedman, L. Eppstein, A. Kadouri, Modified CelliGen-packed bed bioreactors for hybridoma cell cultures, Cytotechnology 9 (1992) 41–49, https://doi.org/10.1007/BF02521730.

[46] S. Guo, X. Zhu, M. Li, L. Shi, O.J.L. T, J. D, N.K. G, Parallel control over surface charge and wettability using polyelectrolyte architecture, effect on protein adsorption and cell adhesion, ACS Appl. Mater. Interfaces. 8 (2016) 30552–30563.

[47] L. Bacakova, E. Filova, M. Parizek, T. Ruml, V. Svorcik, Modulation of cell adhesion, proliferation and differentiation on materials designed for body implants, Biotechnol. Adv. 29 (2011) 739–767, https://doi.org/10.1016/j.biotechadv.2011.06.004.

[48] K. Webb, V. Hlady, P.A. Tresco, Relative importance of surface wettability and charged functional groups on NIH 3T3 fibroblast attachment, spreading, and cytoskeletal organization, J. Biomed. Mater. Res. 41 (1998) 422–430.

[49] A. Bura, V. Planat-Benard, P. Bourin, J.-S. Silvestre, F. Gross, J.-L. Grolleau, B. Saint-Lebese, J.-A. Peyrafitte, S. Fleury, M. Gadelorge, M. Taurand, S. Dupuis-Coronas, B. Leobon, L. Casteilla, Phase I trial: the use of autologous cultured adipose-derived stroma/stem cells to treat patients with non-revascularizable critical limb ischemia, Cytotherapy 16 (2014) 245–257, https://doi.org/10.1016/j.jcyt.2013.11.011.

[50] J. Eom, V. Feisst, L. Ranjard, K. Loomes, T. Damani, V. Jackson-Patel, M. Locke, H. Sheppard, P. Narayan, P.R. Dunbar, Visualization and quantification of mesenchymal cell adipogenic differentiation potential with a lineage specific marker, J. Vis. Exp. (2018), https://doi.org/10.3791/57153.

[51] M.C. Ciuffreda, G. Malpasso, P. Musarò, V. Turco, M. Gnecchi, Protocols for in vitro Differentiation of Human Mesenchymal Stem Cells into Osteogenic, Chondrogenic and Adipogenic Lineages, (n.d.) 10.

[52] Q.A. Rafiq, K. Coopman, A.W. Nienow, C.J. Hewitt, Systematic microcarrier screening and agitated culture conditions improves human mesenchymal stem cell yield in bioreactors, Biotechnol. J. 11 (2016) 473–486, https://doi.org/10.1002/biot.201400862.

[53] P. Silva Couto, M.C. Rotondi, A. Bersenev, C.J. Hewitt, A.W. Nienow, F. Verter, Q. A. Rafiq, Expansion of human mesenchymal stem/stromal cells (hMSCs) in bioreactors using microcarriers: lessons learnt and what the future holds, Biotechnol. Adv. 45 (2020) 107636, https://doi.org/10.1016/j.biotechadv.2020.107636.

[54] I. Sekiya, B.L. Larson, J.R. Smith, R. Pochampally, J.-G. Cui, D.J. Prockop, Expansion of human adult stem cells from bone marrow stroma: conditions that maximize the yields of early progenitors and evaluate their quality, Stem Cells 20 (2002) 530–541, https://doi.org/10.1634/stemcells.20-6-530.

[55] S. Derakhti, S.H. Safiabadi-Tali, G. Amoabediny, M. Sheikhpour, Attachment and detachment strategies in microcarrier-based cell culture technology: a comprehensive review, Mater. Sci. Eng. C 103 (2019) 109782, https://doi.org/10.1016/j.msec.2019.109782.
[56] Y. Li, Q. Wu, Y. Wang, L. Li, H. Bu, J. Bao, Senescence of mesenchymal stem cells (review), Int. J. Mol. Med. 39 (2017) 775–782, https://doi.org/10.3892/ijmm.2017.2912.
[57] M. Dominici, K. Le Blanc, I. Mueller, I. Slaper-Cortenbach, F.C. Marini, D.S. Krause, R.J. Deans, A. Keating, D.J. Prockop, E.M. Horwitz, Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement, Cytotherapy 8 (2006) 315–317, https://doi.org/10.1080/14653240600855905.
[58] P. Bourin, B.A. Bunnell, L. Casteilla, M. Dominici, A.J. Katz, K.L. March, H. Redl, J. P. Rubin, K. Yoshimura, J.M. Gimble, Stromal cells from the adipose tissue-derived stromal vascular fraction and culture expanded adipose tissue-derived stromal/stem cells: a joint statement of the International Federation for Adipose Therapeutics and Science (IFATS) and the International Society for Cellular Therapy (ISCT), Cytotherapy 15 (2013) 641–648, https://doi.org/10.1016/j.jcyt.2013.02.006.
[59] G. Tk, Z. Zy, C. Ak, R. S, C. M, C. Jk, O. Sk, Microcarrier culture for efficient expansion and osteogenic differentiation of human fetal mesenchymal stem cells, Biores. Open Access 2 (2013), https://doi.org/10.1089/biores.2013.0001.
[60] W.S. Hu, J. Meier, D.I. Wang, A mechanistic analysis of the inoculum requirement for the cultivation of mammalian cells on microcarriers, Biotechnol. Bioeng. 27 (May (5)) (1985) 585–595, https://doi.org/10.1002/bit.260270507.
[61] C. Ferrari, F. Balandras, E. Guedon, E. Olmos, I. Chevalot, A. Marc, Limiting cell aggregation during mesenchymal stem cell expansion on microcarriers, Biotechnol. Prog. 28 (2012) 780–787, https://doi.org/10.1002/btpr.1527.
[62] M. Viganò, S. Budelli, C. Lavazza, T. Montemurro, E. Montelatici, S. de Cesare, L. Lazzari, A.R. Orlandi, G. Lunghi, R. Giordano, Tips and tricks for validation of quality control analytical methods in good manufacturing practice mesenchymal stromal cell production, Stem Cells Int. 2018 (2018) 1–16, https://doi.org/10.1155/2018/3038565.
[63] S. Ceccarelli, P. Pontecorvi, E. Anastasiadou, C. Napoli, C. Marchese, Immunomodulatory effect of adipose-derived stem cells: the cutting edge of clinical application, Front. Cell Dev. Biol. 8 (2020) 236, https://doi.org/10.3389/fcell.2020.00236.
[64] K. Ponnuru, J. Wu, P. Ashok, E. Tzanakakis, E. Furlani, Analysis of stem cell culture performance in a microcarrier bioreactor system, in: Technical Proceedings of the 2014 NSTI Nanotechnology Conference and Expo, NSTI-Nanotech 2014, 2, 2014, pp. 132–135.
[65] S. Löffelholz, C. Loffelholz, S. Werner, D. Eibl, CFD for characterizing standard and single-use stirred cell culture bioreactors, in: O. Minin (Ed.), Computational Fluid Dynamics Technologies and Applications, InTech, 2011, https://doi.org/10.5772/23496.
[66] L. Nguyen, S. Bang, I. Noh, Tissue regeneration of human mesenchymal stem cells on porous gelatin micro-carriers by long-term dynamic in vitro culture, Tissue Eng. Regen. Med. 16 (2019) 19–28, https://doi.org/10.1007/s13770-018-00174-8.
[67] A.-C. Tsai, Y. Liu, X. Yuan, R. Chella, T. Ma, Aggregation kinetics of human mesenchymal stem cells under wave motion, Biotechnol. J. 12 (2017) 1600448, https://doi.org/10.1002/biot.201600448.
[68] V. Jossen, C. van den Bos, R. Eibl, D. Eibl, Manufacturing human mesenchymal stem cells at clinical scale: process and regulatory challenges, Appl. Microbiol. Biotechnol. 102 (2018) 3981–3994, https://doi.org/10.1007/s00253-018-8912-x.
[69] F. dos Santos, P.Z. Andrade, M.M. Abecasis, J.M. Gimble, L.G. Chase, A. M. Campbell, S. Boucher, M.C. Vemuri, C.L. da Silva, J.M.S. Cabral, Toward a clinical-grade expansion of mesenchymal stem cells from human sources: a microcarrier-based culture system under xeno-free conditions, Tissue Eng. Part C Methods 17 (2011) 1201–1210, https://doi.org/10.1089/ten.tec.2011.0255.
[70] B. Cunha, T. Aguiar, S.B. Carvalho, M.M. Silva, R.A. Gomes, M.J.T. Carrondo, P. Gomes-Alves, C. Peixoto, M. Serra, P.M. Alves, Bioprocess integration for human mesenchymal stem cells: from up to downstream processing scale-up to cell proteome characterization, J. Biotechnol. 248 (2017) 87–98, https://doi.org/10.1016/j.jbiotec.2017.01.014.
[71] G. Astori, E. Amati, F. Bambi, M. Bernardi, K. Chieregato, R. Schäfer, S. Sella, F. Rodeghiero, Platelet lysate as a substitute for animal serum for the ex-vivo expansion of mesenchymal stem/stromal cells: present and future, Stem Cell Res. Ther. 7 (2016), https://doi.org/10.1186/s13287-016-0352-x.
[72] A.K.-L. Chen, X. Chen, A.B.H. Choo, S. Reuveny, S.K.W. Oh, Critical microcarrier properties affecting the expansion of undifferentiated human embryonic stem cells, Stem Cell Res. 7 (2011) 97–111, https://doi.org/10.1016/j.scr.2011.04.007.
[73] A.K.-L. Chen, S. Reuveny, S.K.W. Oh, Application of human mesenchymal and pluripotent stem cell microcarrier cultures in cellular therapy: achievements and future direction, Biotechnol. Adv. 31 (2013) 1032–1046, https://doi.org/10.1016/j.biotechadv.2013.03.006.