Expansion of Human Mesenchymal Stem Cells on Corning® Synthemax™ II-coated Corning Dissolvable Microcarriers in a Serum-free Cell Culture Medium

Application Note

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Introduction

Human mesenchymal stem cells (hMSCs) are currently the most common adult stem cell type used for cell therapy applications due to their regenerative properties and ability to differentiate into multiple cell lineages (adipocyte, chondrocyte, and osteocyte)[1,2]. Traditionally, hMSCs have been cultured on 2-dimensional cell culture platforms using serum-containing medium. Although these platforms can be used successfully for small-scale expansion of hMSCs, other platforms will be required to generate the quantity of cells required to support the increasing number of clinical trials utilizing hMSCs and the subsequent large-scale production[3]. Microcarriers represent a viable solution by enabling anchorage-dependent stem cells to be cultured in suspension, enabling significant scale-up in bioreactors.

A critical parameter that needs to be considered when selecting the optimal microcarrier is the intended use of the product. For cell therapy applications, a viable, functional cell is the desired product. Unfortunately, most commercially available microcarriers have significant disadvantages that hinder their use for cell therapy applications. Most microcarriers are composed of a solid matrix which necessitates the need for the microcarrier to be separated from the dissociated cells during downstream processing. This separation step adds complexity and expense to the overall production process. Another limitation observed with microcarriers is the difficulty to efficiently dissociate cells following cell expansion due to the strength of cell attachment to the microcarriers. For these microcarriers, overall cell yield is significantly reduced and, more importantly, the functionality of the harvested cells may be impacted by the harsh cell dissociation strategies that are required.

Corning dissolvable microcarriers provide an ideal solution for the large-scale expansion of hMSCs for bioprocess applications. Corning dissolvable microcarriers are composed of cross-linked polysaccharide polymers that can be dissolved during the cell harvest step. The ability to completely dissolve the microcarrier results in simpler downstream purification processes and eliminates the need to physically separate the cells and microcarriers. Corning dissolvable microcarriers are quickly dissolved following the addition of an animal component-free harvest solution. A cell-dissociation enzyme can be added during the harvest step in order to facilitate a single-cell suspension.

Here we describe a user-friendly protocol for the expansion of hMSCs in serum-free medium. hMSCs were cultured on Corning Synthemax™ II-coated dissolvable microcarriers in StemPro® MSC SFM (serum-free medium) demonstrating efficient cell attachment and expansion. Cells were cultured for up to 7 days resulting in cell yields exceeding 40,000 cells/cm^2 (>7-fold expansion). During the cell harvest step, the microcarriers were completely dissolved within 10 minutes resulting in a single-cell suspension. The harvested cells were characterized for marker expression, karyotype, and the ability to differentiate into adipocytes, chondrocytes, and osteocytes. These results demonstrate that Corning dissolvable microcarriers are an ideal scalable platform for the expansion and efficient harvest of functional hMSCs in serum-free medium.

Methods and Materials

Cell Preparation

Human bone marrow-derived mesenchymal stem cells were purchased from RoosterBio (RosterBio Cat. No. MSC-001). Cells were adapted to StemPro MSC SFM (Thermo Fisher Cat. No. A1033201) and expanded in Corning CellBIND® CellSTACK® 2-chamber vessels (Corning Cat. No. 3310) that were coated with Corning Synthemax II-SC at 5 mg/cm^2 (Corning Cat No. 3535). A cell bank was established by cryopreserving the cells in CryoStor® CS5 (STEMCELL Technologies Cat. No. 07933).

To generate cells for microcarrier cultures, hMSCs were thawed and seeded directly into CellBIND CellSTACK 2-chamber vessels (coated with Synthemax II) at 5,000 cells/cm^2 in 300 mL StemPro MSC SFM. The cells were incubated for 5 days in a cell culture incubator with a media exchange (300 mL) on day 3. To harvest, cells were washed once with 100 mL DPBS (Corning Cat. No. 21-031-CM) and then incubated with 50 mL TrypLE™ Select Enzyme (10X) (Thermo Fisher Cat. No. A12177-01) for 8 minutes. Following cell dissociation, cells were centrifuged at 200 xg for 5 minutes and then resuspended in StemPro medium.

Dissolvable Microcarrier Protocols

hMSCs were cultured on Synthemax II-coated Corning dissolvable microcarriers (Corning Cat No. 4983) in StemPro MSC SFM in a 1L Corning ProCulture glass spinner flask (Corning Cat. No. 4500-1L). The final culture volume in each flask was 600 mL. The total microcarrier surface area per flask was 3,000 cm^2 (5 cm^2/mL).

Cells were seeded at a concentration of 6,000 cells/cm^2. Prior to use, the glass spinner flasks were treated with Sigmacote® (MilliporeSigma Cat. No. SL2-25ML), washed with water, and autoclaved to minimize sticking of microcarriers.

Microcarrier Hydration

Corning® dissolvable microcarriers are supplied as a sterile, dry powder and must be hydrated prior to use. To hydrate microcarriers for a single 1L glass spinner flask (GSF), 3,000 cm^2 (0.6 g) dissolvable microcarriers were aseptically transferred to a sterile container. Next, 90 mL sterile water (Corning Cat. No. 25-055-CM) was added to the bottle (150 mL water per gram microcarriers), and the mixture was gently swirled in order to resuspend the microcarriers. For additional information, see Hydration of Dissolvable Microcarriers Protocol (CLS-AN-467). The microcarriers were incubated for 10 minutes to allow complete hydration and then the water was removed from the settled microcarrier bed. **Note:** the microcarriers can be washed in StemPro® MSC SFM in order to minimize dilution of the culture medium due to residual water from the hydration step. Next, the microcarriers were resuspended in StemPro MSC SFM to a final volume of 60 mL (50 cm^2/mL microcarrier concentration).

Preparation of Dissolvable Microcarrier Culture in 1L Glass Spinner Flasks

First, 420 mL of StemPro MSC SFM was added to the 1L GSF. Next, 60 mL of microcarrier suspension (at 50 cm^2/mL) was added to the GSF. The flask was then incubated in a cell culture incubator for ~30 minutes to allow the media to equilibrate. During the equilibration step, hMSCs were harvested from Corning CellBIND® CellSTACK® 2-chamber vessels as described above. Cells were seeded at a density of 6,000 cells/cm^2 ($1.8x10^7$ cells/GSF). The total volume for the cell addition step was 120 mL. If less than 120 mL of cell stock is added to the 1L GSF, the appropriate volume of StemPro MSC SFM should be added to the GSF to bring the total cell addition volume to 120 mL. This will result in a final culture volume of 600 mL for the 1L GSF.

Cell Attachment Phase

An intermittent mixing protocol was employed during the cell attachment phase. After the addition of cells to the 1L GSF, the culture was mixed for 2 minutes at 22 rpm. Next, the culture was incubated at 0 rpm (static phase) for 30 minutes. This process was repeated for 8-12 hours (16-24 cycles). Cell attachment efficiency should exceed 80% at the end of the attachment phase. Cell attachment was monitored by filtering a 1 mL sample of the microcarrier culture through a 35 µm cell strainer (Corning Cat. No. 352235) and quantifying the cell concentration of the flow through.

Cell Expansion Phase

Following the cell attachment phase, the microcarrier culture was mixed continuously at 16 rpm. During the cell expansion phase, the mixing speed was increased daily in an attempt to minimize microcarrier aggregation. The mixing speed was increased as follows: 16 rpm (following cell attachment), 16 rpm (day 1), 22 rpm (day 2), 27 rpm (day 3), 32 rpm (days 4-7). Half-volume media exchanges were performed on days 3 and 5. To exchange the media, the microcarriers were allowed to settle in the 1L GSF for 3-5 minutes. Next, 300 mL of the spent media was removed and 300 mL fresh StemPro MSC SFM was added. The 1L GSF was then returned to the stir platform in the cell culture incubator.

Microcarrier Dissolution and Cell Release

Cells were harvested from the dissolvable microcarriers after 5 to 7 days of expansion. First, a "harvest solution" was prepared in order to fully dissolve the microcarriers and release the attached cells. We recommend adding 250 mL harvest solution per gram of dry microcarriers. The recommended harvest solution for hMSCs is: 100 U/mL pectinase (MilliporeSigma Cat. No. P2611), 10 mM EDTA (Corning Cat. No. 46-034-CI), 5X TryPLE™ Select (Thermo Fisher Cat. No. A12177-01) diluted in DPBS (Corning Cat. No. 21-031-CM). The harvest solution was filter-sterilized (Corning Cat. No. 430767) and pre-warmed to 37°C prior to use.

To begin the harvest process, the 1L GSF was removed from the stir platform and allowed to incubate for 3-5 minutes in order to allow the microcarriers to settle. Next, the spent media was removed and the microcarriers were washed with 200-300 mL DPBS (Corning Cat. No. 21-031-CM). The microcarriers were allowed to settle after the addition of DPBS and the supernatant was then removed. To dissolve the microcarriers, 150 mL of harvest solution was added to the 1L GSF and the flask was incubated on a stir platform for 10-15 minutes at 32 rpm. Once a single-cell suspension was observed, the cells were centrifuged at 200 xg for 5 minutes in a 250 mL centrifuge tube (Corning Cat. No 430776) and then resuspended in CryoStor® CS5 for cryopreservation.

Cell Characterization

To demonstrate that the hMSCs maintained multipotency after expansion on Corning dissolvable microcarriers, the harvested hMSCs were differentiated into adipocytes, chondrocytes, and osteocytes. First, the cells were thawed and washed in culture medium to remove the freezing medium. For adipocyte and osteocyte differentiation, the cells were re-seeded in Corning CellBIND surface 6-well plates (Corning Cat. No. 3335). For chondrocyte differentiation, the cells were re-seeded in Tissue Culture (TC)-treated 6-well plates (Corning Cat. No. 3506). The cells were differentiated according to manufacturer's recommendations (Thermo Fisher Cat. Nos. A10070-01, A10072-01, and A10071-01). After 2-3 weeks, differentiated cells were fixed in 2% paraformaldehyde and stained for characteristic markers of differentiation: adipocytes (Oil Red O; MilliporeSigma Cat. No. O1516-250ML), osteocytes (Alizarin Red; MilliporeSigma Cat. No. TMS-008-C), and chondrocytes (Alcian Blue; MilliporeSigma Cat. No. TMS-010-C).

For quantitative analysis of cell phenotype, cells were thawed and immunostained for positive and negative surface markers. Positive markers included CD73 (BD Biosciences Cat. No. 550256), CD90 (BD Biosciences Cat. No. 555593), CD105 (BD Biosciences Cat. No. 555690). Negative markers included CD14 (MilliporeSigma Cat. No. MAB1219) and CD34 (BD Biosciences Cat. No. 555820). Cells were replated overnight on T-175 flasks (CellBIND surface, coated with Corning Synthemax™ II) to acquire CD105 data. Flow cytometry was used to quantify the percentage of viable cells expressing each marker. Briefly, cells were

diluted to $1x10^6$ cells/mL in 1X DPBS (Corning Cat. No. 21-031) supplemented with 10% heat-inactivated serum (Corning Cat. No. 35-011-CV) (blocking buffer) and 1:1000 dilution of propidium iodide (MilliporeSigma Cat. No. P4864). The cells were then incubated in the dark for 20 minutes at room temperature. Cells were washed twice with 1X DPBS, resuspended in blocking buffer at $0.5x10^6$ cells/50 µL, and incubated with primary antibodies (10 µg/mL) or corresponding IgG1 isotype control (BD Biosciences Cat. No. 554121) for 30 minutes at 4°C in the dark. Cells were washed in staining buffer containing BSA (BD Biosciences Cat. No. 554657) and then incubated with secondary antibody (1:1000 dilution of Alexa Fluor® 488 goat anti-mouse IgG1 [Thermo Fisher Cat. No. A11001]) for 30 minutes at 4°C in the dark. Stained cells were washed once and resuspended in staining buffer for processing on a BD FACSCalibur™ flow cytometer. During acquisition, 30,000 events/sample were collected and analyzed using the BD CellQuest™ Pro software using dot plot analysis with double gating for FSC/SSC and FL3-PI- vs. PI+, live vs. dead cells.

For analysis of cell karyotype, cells were submitted as live cultures for G-banding karyotype analysis (WiCell Research Institute, Cytogenetics Laboratory).

Results

Attachment of hMSCs on Corning® Synthemax™ II-coated Dissolvable Microcarriers

Cell attachment to microcarriers was monitored by quantifying the number of unattached cells in the culture medium for up to 24 hours after cell seeding. Figure 1 shows images of the microcarrier cultures at the time of cell seeding (0 hours) and 24 hours post-cell seeding. By the end of the attachment phase, nearly all of the cells were attached to the microcarriers resulting in an attachment efficiency of 80% to 90%. Importantly, the cells demonstrated uniform attachment to the microcarriers with minimal bare microcarriers observed. Additionally, the cells exhibited the expected cell morphology after the cell attachment phase, which suggests that the microcarrier surface and cell attachment conditions were appropriate.

Expansion of hMSCs on Corning Synthemax II-coated Dissolvable Microcarriers

Cells were expanded on Corning dissolvable microcarriers for 7 days with half volume media exchanges every 2-3 days. Figure 2 shows microscope images of the microcarrier culture during the expansion phase. A uniform distribution of cells was observed for the first 2-3 days of the culture with the cells continuing to demonstrate the expected cell morphology. At the latter phase of the culture period, the cells achieved confluency with the concurrent appearance of microcarrier aggregates. The mixing speed was increased daily during the expansion phase in an attempt to minimize aggregation of the microcarriers; however, a more aggressive mixing strategy may be required to further reduce microcarrier aggregation. Microcarrier aggregation has previously been reported for hMSCs cultured in serum-free medium on other microcarriers[4].

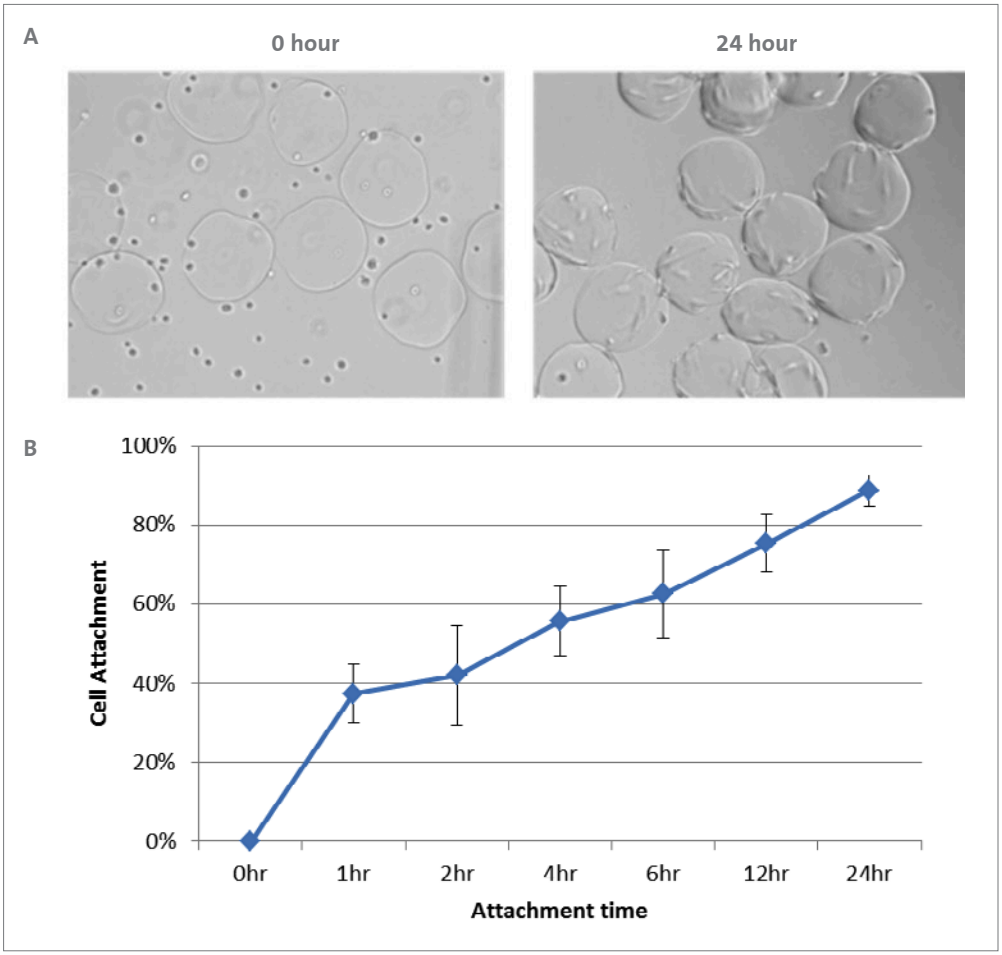

Figure 1. Demonstration of hMSC attachment to Corning Synthemax II-coated dissolvable microcarriers. Panel A: Representative microscope images of hMSCs and Synthemax II-coated dissolvable microcarriers during the cell attachment phase at 0 hours (left panel) and 24 hours (right panel). 10X magnification. Panel B: Cell attachment efficiency for hMSCs on Synthemax II-coated dissolvable microcarriers (n=3). Cell attachment efficiency was determined by quantifying the number of unattached cells in the culture.

Microcarrier Dissolution and Harvest of hMSCs

Cells were harvested from 1L GSFs on days 5, 6, and 7. Figure 3 shows that complete microcarrier dissolution was observed 2-4 minutes after addition of the harvest solution. The harvest phase was allowed to continue for 10-20 minutes in order to generate a single-cell suspension. The total harvest time is dependent upon the level of confluency of the microcarrier culture. Less time may be required for subconfluent microcarriers, whereas longer times may be required for confluent microcarriers.

Figure 4 shows the overall cell yield for each culture following microcarrier dissolution and cell release. On day 5, cell concentrations of 25,000 cells/cm^2 were achieved corresponding to 4-fold expansion. By day 7, cell concentrations routinely exceeded 40,000 cells/cm^2 (>7-fold expansion) with high viability (>90%). Due to the ability to completely dissolve the microcarriers, cell recovery can be significantly improved compared to other commercially available microcarriers.

Characterization of hMSCs Harvested from Corning® Dissolvable Microcarriers

hMSCs harvested from the dissolvable microcarriers were fully characterized for cell marker expression, karyotype, and the ability to differentiate into adipocyte, chondrocyte, and osteocyte cell

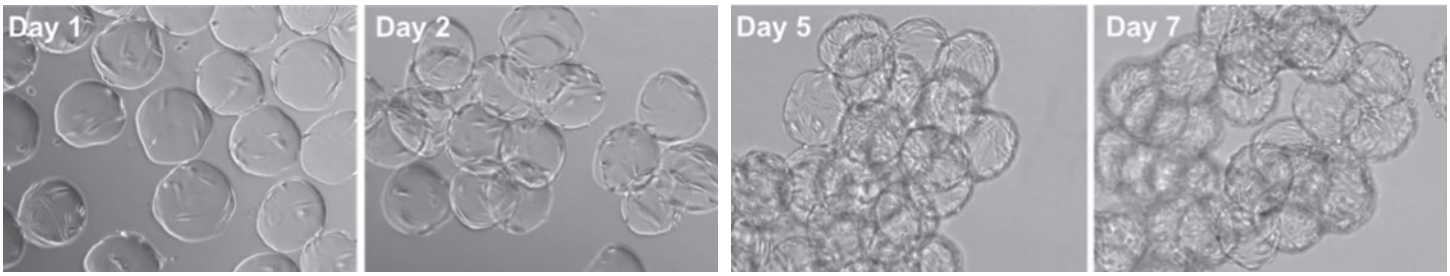

Figure 2. Images of cell expansion on Corning Synthemax™ II-coated dissolvable microcarriers. Representative microscope images of hMSCs on Synthemax II-coated dissolvable microcarriers on days 1, 2, 5, and 7 (10X magnification).

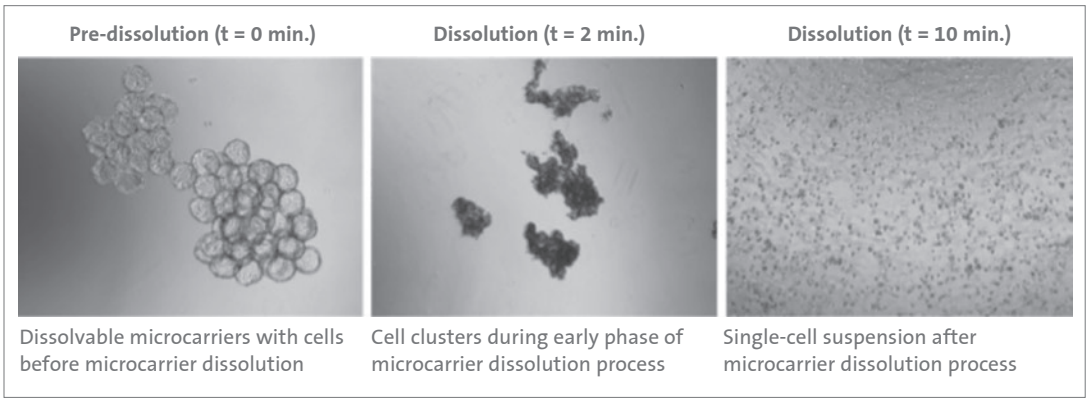

Figure 3. Microcarrier dissolution and cell release. Representative microscope images at different time points during the cell harvest phase (4X magnification). Microcarriers were dissolved in a 1L glass spinner flask following the addition of a harvest solution consisting of pectinase, EDTA, and TrypLE™. The microcarrier culture was mixed at 32 rpm during the harvest phase.

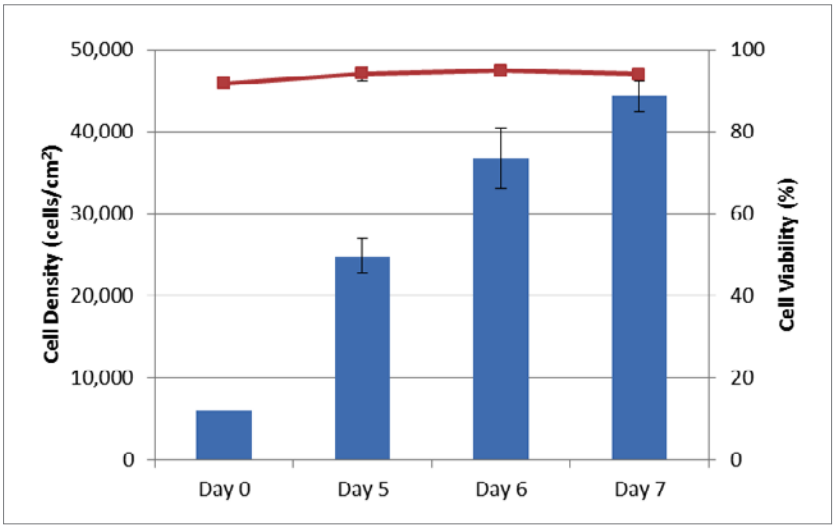

Figure 4. Quantitation of cell yield and cell viability following microcarrier dissolution and cell release. The entire microcarrier culture was harvested from individual 1L glass spinner flasks on days 5, 6, and 7. Following microcarrier dissolution and cell release, cells were quantified on a Vi-CELL™ automated cell counter. Blue bars represent cell concentration (cells/cm^2); red line represents cell viability following cell harvest (n = 2, Days 5 and 6; n = 3, Day 7).

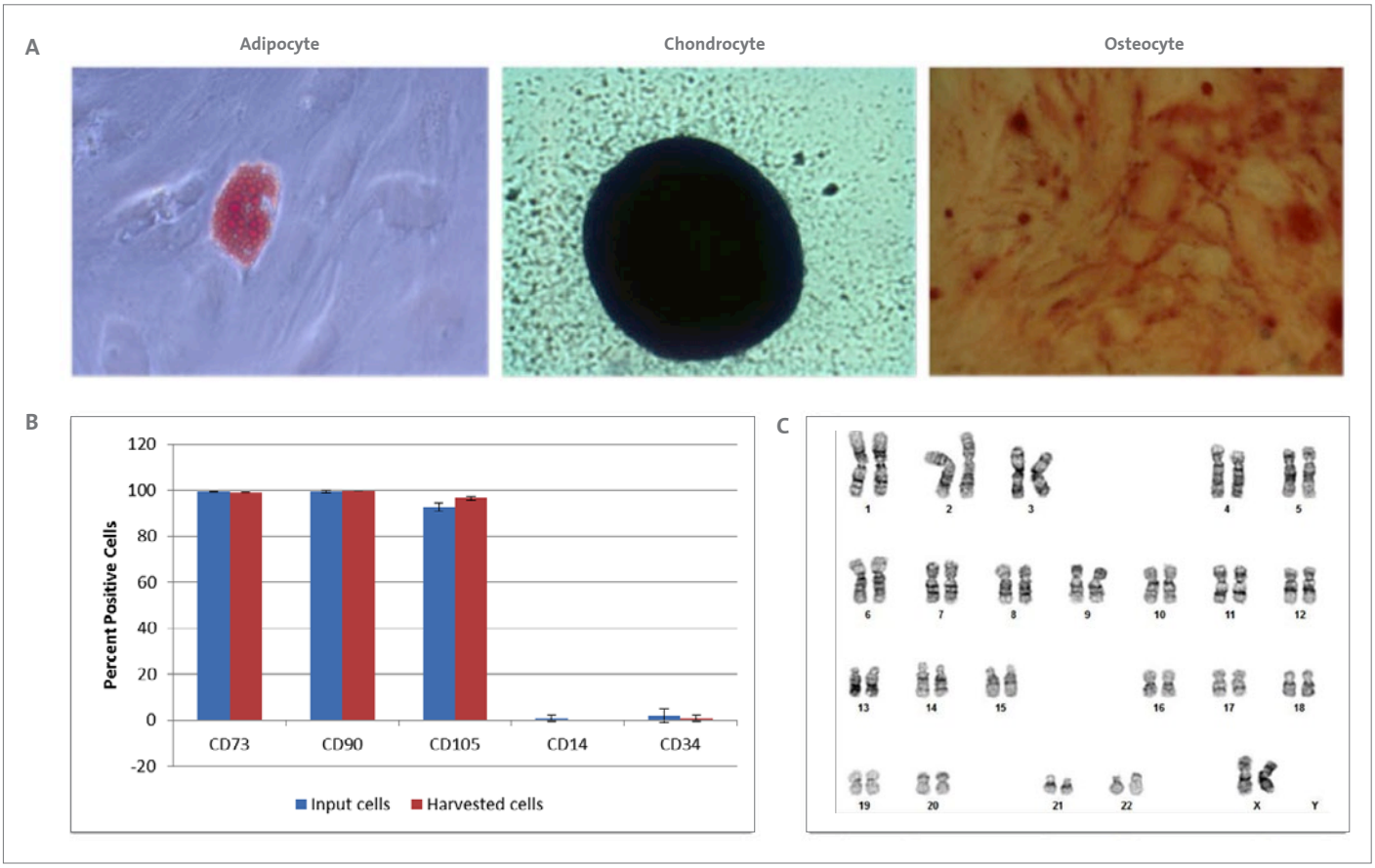

Figure 5. Characterization of hMSCs harvested from Corning Synthemax II-coated dissolvable microcarriers. Cells harvested from dissolvable microcarriers were characterized for 1) ability to differentiate into adipocytes, chondrocytes, and osteocytes; 2) expression of cell surface markers; and 3) genetic stability. Panel A: Confirmation of terminal differentiation of adipocyte, chondrocyte, and osteocyte cell lineages following incubation with the appropriate differentiation medium for 14-21 days. Cell differentiation was confirmed by staining with Oil Red O (adipocyte), Alcian Blue (chondrocyte), and Alizarin Red S (osteocyte). Adipocyte (20X magnification), chondrocyte, and osteocyte (2.5X magnification). Panel B: Representative flow cytometry data for specific cell surface markers (n = 2). Input cells represent cells that were used to seed the dissolvable microcarriers. Panel C: Demonstration of normal karyotype for hMSCs harvested from dissolvable microcarriers. Live cultures were submitted for G-banded karyotype analysis.

lineages (Figure 5). Terminal differentiation into adipocyte, chondrocyte, and osteocyte lineages was confirmed using traditional staining methods for characteristic markers. Flow cytometry confirmed the expression of the positive surface markers CD73, CD90, and CD105 and the absence of CD14 and CD34. Chromosome analysis confirmed that the cells maintained the normal karyotype. The characterization results demonstrate that hMSCs expanded and harvested from dissolvable microcarriers maintain their phenotype, multipotency, and genetic stability.

Summary/Conclusions

- Novel cell culture platforms are needed to meet the expected future demand of hMSCs that is being driven by the emergence of regenerative medicine and cell-based therapy. Existing cell culture platforms have limitations that hamper their ability to generate sufficient quantities of therapeutically active cells using well-controlled processes. Corning® dissolvable microcarriers provide a scalable solution for the large-scale expansion and harvest of functional hMSCs by enabling simplified downstream processing and high yield cell recovery.
- A user-friendly protocol was developed to efficiently expand hMSCs on Corning Synthemax™ II-coated dissolvable microcarriers in a serum-free medium.
- Complete dissolution of the microcarriers was observed during the harvest phase resulting in high recovery of functional hMSCs.
- hMSCs harvested from dissolvable microcarriers maintained multipotency and demonstrated the expected phenotype.
- Corning Synthemax™ II-coated dissolvable microcarriers provide an ideal platform for the scale-up and harvest of functional hMSCs for bioprocess applications.

References

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