Large Scale Expansion of Human Mesenchymal Stem Cells using Corning® stemgro® hMSC Medium and Corning CellBIND® Surface HYPER*Stack*® Cell Culture Vessels

Application Note

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Introduction

Mesenchymal stem cells (MSCs) are multipotent cells that exhibit the capacity to differentiate into multiple cell lineages such as osteoblasts, chondrocytes and adipocytes. The ability to self-renew and differentiate under appropriate culture conditions not only makes MSCs valuable for T-cell therapy and tissue engineering applications, but also drives the demand for large scale expansion. Traditional MSC culturing methods involve the use of serum-containing media, but the undefined nature of serum is a concern in clinical applications. While chemically defined, serum-free media formulations are available[1], many of these media require pre-coating of the growth surface with biological substrates. Biological coating of growth surfaces may be both challenging and expensive during large scale production.

Corning stemgro hMSC Medium is a chemically defined, serum-free medium formulated for maximum expansion of hMSCs. When used in conjunction with the Corning CellBIND Surface, stemgro hMSC Medium enables attachment and growth comparable to that of serum-containing cultures, including maintenance of MSC multipotency[2], but eliminates the need for biological coatings.

Corning CellBIND Surface HYPER*Stack* Cell Culture Vessels (Fig. 1) use the patented HYPER technology platform, consisting of a gas permeable film to eliminate the requirement for the air gap normally found in standard cell culture vessels. This allows for a compact vessel with increased growth area. The HYPER*Stack*-12 layer (6,000 cm^2, A) and 36 layer (18,000 cm^2, B) vessels are designed for closed system applications and are manufactured using methods acceptable for large-scale cell propagation.

The following study demonstrates the large scale expansion of hMSCs in a serum-free environment using stemgro hMSC Medium in combination with HYPER*Stack*-12 Layer Cell Culture Vessels. To monitor the health and functionality of the hMSC cultures, cell yields, viability and surface antigen profiles (positive antigens: CD105, CD166 and CD29 and negative antigens: CD14 and CD34) were assessed throughout the scale up process. To confirm the functionality of expanded cultures of hMSCs, the cells were harvested from HYPER*Stack*-12 Vessels and induced to differentiate into adipogenic and chondrogenic lineages.

Methods and Materials

Cell Cultures

Bone marrow-derived human mesenchymal stem cells; hMSC (STEMCELL™ Technologies [SCT] Cat. No. MSC-001F, Lot No. BM2758) were thawed and maintained in complete stemgro hMSC Medium (Corning Cat. No. 40-410-KIT) supplemented with 2 mM L-glutamine, (Corning Cat. No. 61-030-RM). Freeze banks of 1.0 x 10^6 cells/vial (passage #2) were prepared using 95% complete growth medium and 5% DMSO.

Thaw and Maintenance

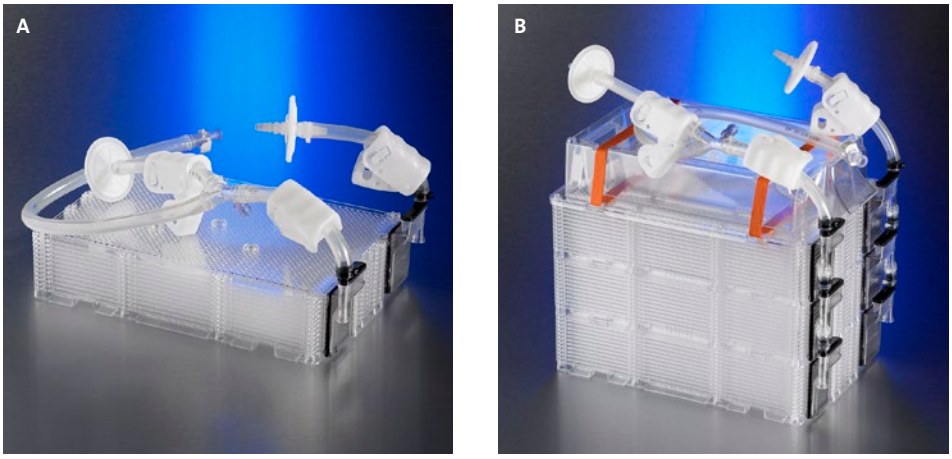

Figure 1. Corning CellBIND Surface HYPER*Stack* Cell Culture Vessels product line. (A) 12-layer and (B) 36-layer.

Frozen hMSC stocks were thawed using standard cell culture protocols. Freeze media was removed by centrifugation at 500 x g for 4 minutes and replaced with fresh growth medium. Cells were plated at a concentration of 5,000 cells/cm^2 on Corning® CellBIND® Surface flasks and designated passage #0. Cells were maintained in cultured until they reached 75 to 80% confluence (4 to 5 days) in a humidified incubator set to 37°C and 5% CO_2. A full medium change was performed a minimum of 24 hours prior to harvest by aspirating spent medium from the vessel and replacing with freshly prepared room temperature growth medium.

Subculture and Scale-up

On the day of harvest (75 to 80% confluent cultures) medium was aspirated from the vessel followed by a gentle rinse using 1x HBSS (Corning Cat. No. 21-023-CM). To maintain serum-free conditions, cultures were harvested with TrypLE™ Express (Invitrogen™ Cat. No. 12605-028) cell dissociation reagent. The TrypLE solution was added to culture vessels using approximately 0.03 mL of reagent per cm^2 and incubated at room temperature for 3 to 5 minutes. After most of the cells were released from the growth surface, the cell suspension was collected into an equal volume of complete growth medium. The empty vessel was further incubated at room temperature for approximately 3 more minutes to allow the remaining cells to detach. Cells were collected from the vessel with an HBSS rinse and pooled with the initial collection. When needed, a second rinse was done using complete growth medium. The cell suspension was concentrated by centrifugation at 500 x g for 5 to 10 minutes (centrifugation time based upon volume of cell collection, see table 1) and resuspended in fresh medium. Cell concentration and viability were assessed using a BioProfile® FLEX instrument (Nova Biomedical). Cells were plated at a concentration of 5,000 cells/cm^2 into larger surface area vessels such as the Corning CellBIND Surface HYPER*Flask*® M Cell Culture Vessel, 1,720 cm^2 (Corning Cat. No. 10034) or Falcon® Cell Culture Multi-Flask, 525 cm^2 (Corning Cat. No. 353143). Scale up continued until enough cells were collected to seed a HYPER*Stack*®-12 Layer Cell Culture Vessel (Corning Cat. No. 10012) and a T-175 Corning CellBIND Surface control flask (Corning Cat. No. 3292) at a concentration of 5,000 cells/cm^2 in 0.217 mL/cm^2 complete Corning stemgro® hMSC Medium. At the end of each harvest a small sample of cell suspension (1 to 5 mL) was saved for phenotypic analysis of cells. The study was independently repeated four times.

Morphologic and Proliferation Assessment

To monitor the general health of the hMSC cultures throughout the scale up process, population doublings and morphologic changes were assessed via microscopic examination using an inverted Olympus microscope. Population doublings were assessed using the equation n = 3.32 (logN-logX); where n = population doubling, N = total number of cells harvested and X = initial number of cells plated.

Immunophenotyping Assessment

Surface marker analysis of the hMSCs were performed throughout each cell passage in order to monitor potential changes to the cells. Cell samples were washed twice (600 x g for 5 minutes) with 5 mL of flow cytometry staining buffer (SB) (R&D Systems® Cat. No. FC001) to remove residual culture medium. Cells were fixed by resuspending cell pellets at a concentration of 5.0 x 10^6 cells/mL in a 1:1 mixture of SB and 4% paraformaldehyde (PFA) (Boston BioProducts Cat. No. BM-155-250 mL) and stored at 4°C for up to 48 hours. (Note: a marked decrease in CD29+ expression was observed if the sample was not analyzed within 48 hours after collection. All other markers remained stable for up to 5 days in storage.)

On the day of analysis, the fixed samples were pelleted by centrifugation at 600 x g for 5 minutes to remove fixing solution, resuspended in blocking buffer (0.5% BSA in staining buffer) to a density of approximately 10 x 10^6 cells/mL and incubated for 15 minutes at room temperature. After blocking, 100 µL of samples (~1.0 x 10^6 cells/mL) were aliquoted into 2 mL tubes and marked as follows: no stain control, IgG1 isotype control, CD105+, CD166+, CD29+, CD14- and CD34- (R&D Systems Cat. Nos. IC002P, FAB10971P, FAB6561P, FAB17781P, FAB3832P, and FAB7227P, respectively). Ten microliters of the corresponding conjugated antibody or isotype control were then added to each sam-

Table 1. Reagent volumes and centrifugation times. Reagent and media volumes used during expansion procedure as well as centrifugation times and speeds.

	Falcon Multi-Flask (525 cm^2)		Corning CellBIND Surface HYPER*Flask* Vessel (1,720 cm^2)		Corning CellBIND Surface HYPER*Stack*-12 Vessel (6,000 cm^2)	
	mL/cm^2	mL	mL/cm^2	mL	mL/cm^2	mL
HBSS Rinse	0.03	15	0.03	50	0.03	200
TrypLE	0.03	15	0.03	50	0.03	200
Quench Media	0.03	15	0.03	50	0.03	200
HBSS Rinse	0.03	15	0.03	50	0.03	200
Media Rinse	0.03	15	0.03	50	0.03	200
Total Volume		60		200		800
Centrifuge Tube		2 x 50 mL		250 mL		2 x 500 mL
Centrifuge Time (@ 500 x g)		5 min		10 min		15 min

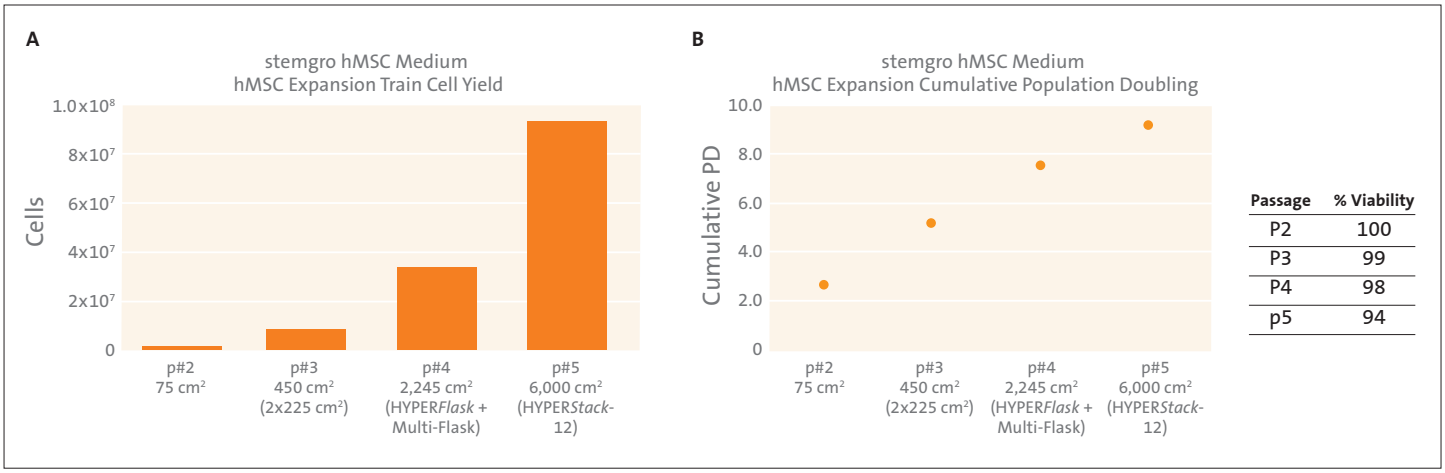

Figure 2. hMSC Expansion. Corning® stemgro® hMSC Medium supported expansion of hMSC cultures (a) while maintaining consistent population doublings (b) and cell viability (c). A representative example of typical seed train (data from first expansion study).

ple tube, mixed well and incubated for 30 to 45 minutes at room temperature while being protected from light. After incubation, cells were washed and pelleted by centrifugation 2 times using SB to remove unbound antibody (4 minutes at 300 x g). Cell pellets were resuspended in 200 to 500 µL of SB (volume based on size of cell pellet) for flow cytometry analysis using a MACSQuant® Analyzer (Miltenyi Biotec).

Multipotency Assessment

To evaluate whether multipotency was maintained throughout the course of the expansion, hMSCs harvested from Corning® CellBIND® Surface HYPER*Stack*®-12 Vessels and Corning CellBIND Surface T-175 control flask were differentiated into adipocytes and chondrocytes. Adipogenic Differentiation Bullet Kit® (Lonza Cat. No. PT-3004) was utilized for adipogenic differentiation of hMSCs. Following the kit's protocol, hMSCs were plated at a concentration of 21,000 cells per cm^2 in Corning CellBIND Surface 12 Well Multiple Well Plates (Corning Cat. No. 3336) and maintained in complete stemgro hMSC Medium until they were ready to differentiate using the kit's induction medium. Adipocyte differentiation was confirmed through Oil Red O Staining and quantitated using AdipoRed™ Assay Reagent (Lonza Cat. No. PT-7009) and analyzed on an EnVision® Microplate Reader (PerkinElmer). StemPro® Chondrogenesis Differentiation Kit (Life Technologies™ Cat. No. 10071-01) was used to induce chondrogenic differentiation. Following the manufacturer's protocol, hMSCs were suspended in complete stemgro hMSC Medium at a concentration of 1.6 x 10^7 cells/mL and plated on Corning CellBIND Surface 12 Well Multiple Well Plates by placing a 5 µL droplet in the middle of each well. Differentiation followed using the kit's induction media as follows: after a 2-hour incubation under high humidity, 1 mL of chondrogenic or control media (stemgro hMSC Medium) was added to the wells. The cells were then cultured at 37°C in 5% CO_2 for up to 3 weeks with complete media changes every 3 days. Following 22 days in culture, the Alcian Blue Stain (Newcomer Supply® Cat. No. 1003) was added to confirm chondrocyte formation.

Results

The International Society for Cellular Therapy lists the following criteria for defining functional MSCs:

- Attach and proliferate *in vitro* under standard culture conditions
- Maintain phenotypical characterization through the expression of surface antigens, and
- Differentiate along the adipogenic, chondrogenic and osteogenic lineages[3]. These criteria were applied to evaluate the quality of the hMSCs that were cultured and expanded in stemgro hMSC Medium and on Corning HYPER technology vessels.

Proliferation

The effectiveness of the stemgro hMSC Medium as a serum-free medium for the expansion of hMSCs without the need for biological coatings was assessed by the healthy scale up of the hMSCs into the HYPER*Stack*-12 vessel. A primary metric of this study was to maintain the multipotency of the hMSCs. For this study, the hMSC cultures were maintained at a low passage and at approximately 75 to 80% confluence throughout the scale-up to the HYPER*Stack*-12 vessel to conserve the multipotency of the cells. The stemgro hMSC Medium supported the expansion of hMSC cultures from thaw to HYPER*Stack*-12 vessels in 3 to 4 passages without effect on rate of cell growth. Figure 2 shows a representative example of a typical expansion process. hMSCs were thawed and seeded at a concentration of 5,000 cells per cm^2. Cultures were then sequentially scaled into larger surface area vessels using the same seeding concentration until reaching the HYPER*Stack*-12 Vessel (6,000 cm^2) seeding density. Population doublings were calculated after each harvest using the equation n = 3.32 (logN–logX). The results demonstrate consistent population doublings throughout the length of the expansion process. Cell viability, determined by Trypan blue exclusion (BioProfile® Flex analyzer) also indicated a healthy viable cell population.

Expansion Yields

Expansion of hMSCs into the Corning® CellBIND® Surface HYPER*Stack*®-12 and Corning CellBIND Surface T-175 control vessels was the final scale up step in this study. Vessels were seeded at a concentration of 5,000 cells/cm^2 in complete Corning stemgro hMSC Medium (0.217 mL/cm^2) and cultured for 4 days (75 to 80% confluence). Cells were harvested with TrypLE reagent and enumerated using the Trypan blue exclusion method.

Viable cell counts from four independent studies show average yields of 1.17 x 10^8 cells from the HYPER*Stack*-12 vessels with viabilities of 92% or better (Fig. 3), demonstrating a suitable expansion process. Cells harvested from the T-175 control flasks were compared to those harvested from HYPER*Stack*-12 vessels to evaluate proliferation on the Corning CellBIND Surface treated gas-permeable film. The results demonstrated no statistical difference between cell yields per growth area (cm^2) (paired *t*-test value 0.1148) among the two vessel types (Fig. 4). The data demonstrate not only comparable cell growth between vessels, but also the ability to expand hMSCs. Each expansion study was independently repeated four times.

Phenotypical Characterization

Cell morphology and immunophenotyping of the hMSCs were indicators of normal cell function throughout the expansion process. Microscopic examination revealed no morphologic changes during the scale up process or the final expansion step. Figure 5 demonstrates the similarities in morphology and confluence of hMSC cultures during different stages of the scale up process.

In addition to cell expansion and morphology, immunophenotyping was performed to further evaluate the hMSC cultures. Samples from each scale-up step were collected and analyzed for the positive and negative expression of surface antigens by flow cytometry analysis. CD105, CD166 and CD29 markers were the positive expression markers while CD14 and CD34 were the negative expression markers. These markers were chosen based on the analysis report generated by STEMCELL™ Technologies. Flow cytometric analysis demonstrated consistent expression of the various surface markers throughout the length of each expansion, with >99% expression for each of the positive markers and <5% expression for the negative markers (Fig. 6 and Table 2).

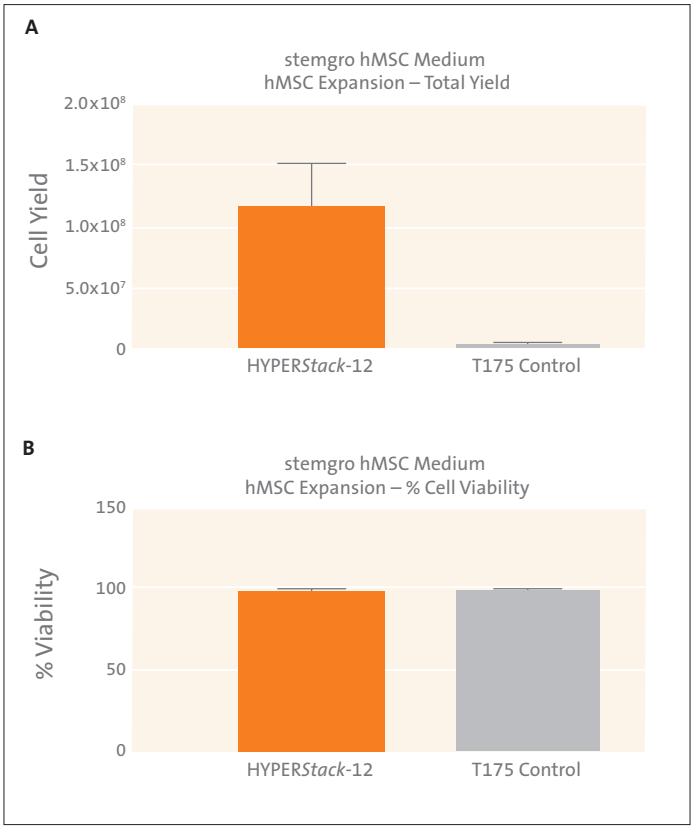

Figure 3. hMSC expansion yields. Final expansion of hMSC in Corning CellBIND Surface HYPER*Stack*-12 Layer Cell Culture Vessels using Corning stemgro hMSC Medium. (A) Average cell yields and (B) average percent cell viability from the four independent studies.

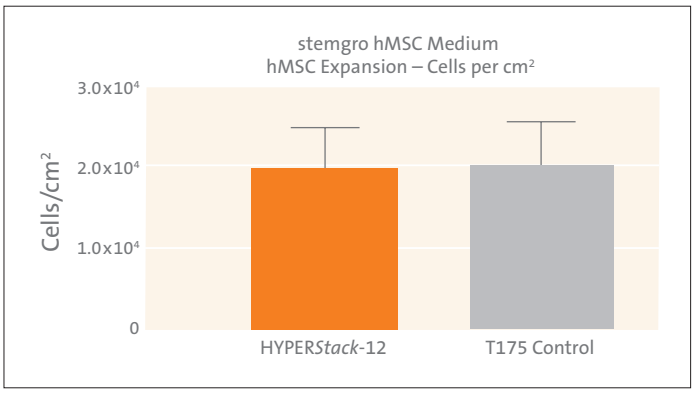

Figure 4. hMSC expansion yields in cells/cm^2. No statistical significant difference was observed between the Corning CellBIND Surface HYPER*Stack*-12 Vessels and T-175 Corning CellBIND Surface control flask yields (n=4; paired *t*-test value: p<0.05).

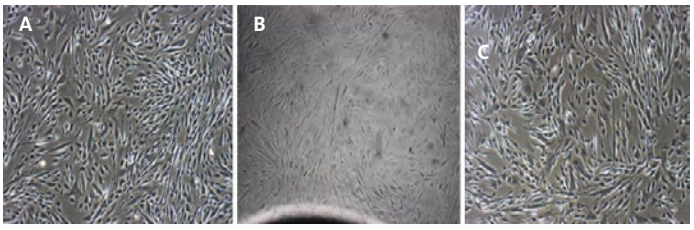

Figure 5. Morphological evaluation of hMSC cultures. Representative micrographs of hMSCs cultured in Corning stemgro hMSC Medium. (A) Vendor hMSC stocks (psg #1) 96 hour cultures on a T-225 Corning CellBIND Surface flask. (B) hMSC cultures at passage #5 on a Corning CellBIND Surface HYPER*Stack*-12 Vessel and (C) T-175 Corning CellBIND Surface control flask (magnification = 4x using inverted Olympus microscope).

Differentiation

The ability to differentiate into multiple cell lineages is an important property of hMSCs. The differentiation potential of hMSCs cultured in Corning® stemgro® hMSC Medium was assessed after expansion in the Corning CellBIND® Surface HYPER*Stack*®-12 Vessel. The hMSCs were differentiated into adipocytes and chondrocytes using commercially available kits. Intracellular triglyceride droplets were used as markers to help identify MSC differentiation into adipocytes. The lipid droplets (red) were stained with Oil Red O using the AdipoRed™ assay for visual confirmation of adipocyte differentiation (Fig. 7). The AdipoRed assay was performed to quantitate adipocyte differentiation of cells harvested from HYPER*Stack*-12 Vessels and T-175 control flasks. Results indicated that cells derived from HYPER*Stack* Vessels had a significant increase (paired *t*-test value of 0.003) in adipocyte differentiation over cells derived from T-175 control vessels (Fig. 8). Further studies of the statistical difference in adipocyte differentiation between both vessel types are ongoing.

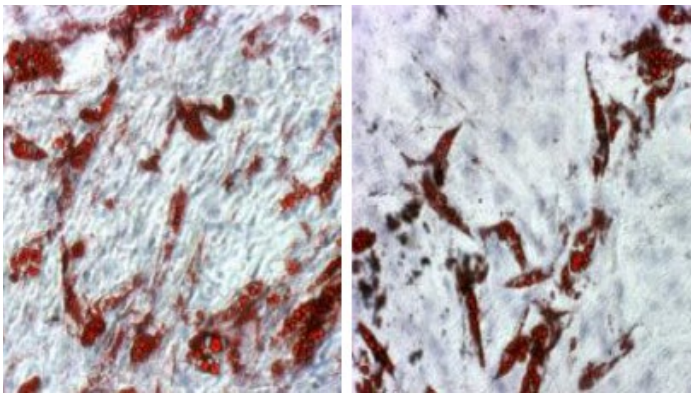

Figure 7. Oil Red O stain of adipocytes. Representative example of adipocyte differentiation of hMSCs. Intracellular lipid droplets stained red with Oil Red O stain. Micrographs of differentiated hMSCs from third expansion study; Corning CellBIND Surface HYPER*Stack*-12 Vessel (left) and T-175 Corning CellBIND Surface control flask (right) derived, (magnification = 10x using inverted Olympus microscope).

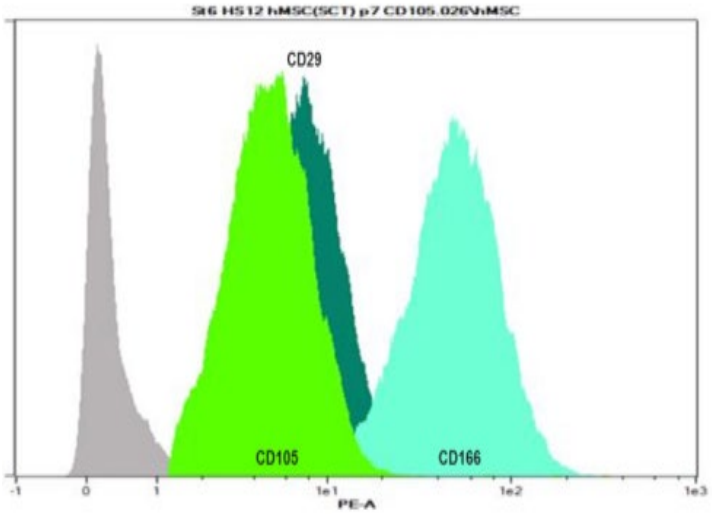

Figure 6. Representative flow cytometry analysis. Flow cytometry histograms of hMSCs harvested from Corning CellBIND Surface HYPER*Stack*-12 Vessel (second expansion study, passage #7). Positive expression response to CD105, CD166 and CD29 surface markers, gray peak is indicative of the isotype control.

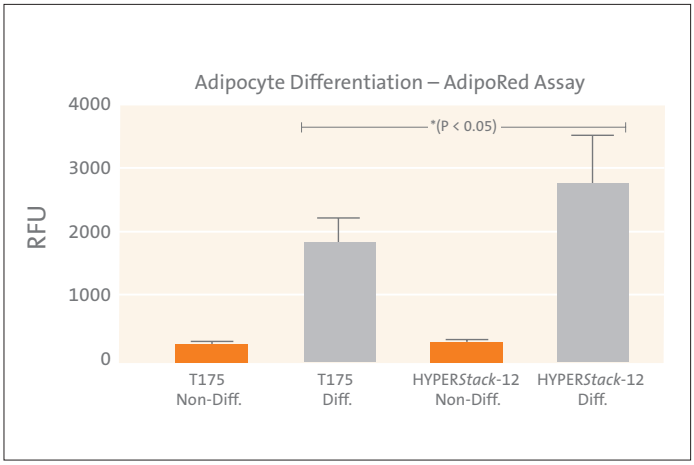

Figure 8. Adipogenic assessment. The AdipoRed™ assay was performed to quantitate adipocyte differentiation. Results indicated statistically higher (paired *t*-test value $p<0.05$) differentiation of cells derived from Corning CellBIND Surface HYPER*Stack*-12 Vessel compared to cells derived from T-175 Corning CellBIND Surface control flask. Results are the average from four independent studies, 3 wells per differentiation condition (n = 12 wells/4 studies).

Table 2. Immunophenotyping assessment of hMSC cultures. Surface marker expression analysis indicated no changes to hMSC line throughout the expansion process. Purity of cells from each expansion was comparable, or better than, the original vendor stock.

		Expansion #1		Expansion #2		Expansion #3		Expansion #4	
		Corning CellBIND Surface		Corning CellBIND Surface		Corning CellBIND Surface		Corning CellBIND Surface	
Culture	Stock Lot Analysis	T-175	HYPER*Stack*-12	T-175	HYPER*Stack*-12	T-175	HYPER*Stack*-12	T-175	HYPER*Stack*-12
IgG2 Iso		0.7%		0.4%		14.0%		0.3%	
CD105	83.25%	97.9%	98.1%	97.6%	97.2%	98.6%	99.1%	99.5%	99.5%
CD166	86.17%	98.7%	99.9%	99.7%	99.7%	99.8%	99.9%	99.9%	99.9%
CD29	92.98%	N/A	N/A	98.8%	99.5%	99.8%	99.8%	99.9%	100.0%
CD14	3.36%	2.2%	0.4%	1.2%	0.4%	0.8%	0.3%	1.5%	0.4%
CD34	0.00%	1.2%	0.5%	2.3%	1.1%	0.2%	0.2%	1.3%	1.0%

For additional verification, hMSCs cultured in the Corning® CellBIND® Surface HYPER*Stack*®-12 Vessels were also induced to differentiate into chondrocytes using the StemPro® Chondrocygenesis Differentiation kit (Life Technologies™). When MSCs are differentiated into chondrocytes, the cells form a micro-mass of chondrogenic (cartilage) cells. This mass will begin to synthesize extracellular matrix (ECM) proteins such as proteoglycans. To visualize differentiation, Alcian Blue stain was applied to the micro-mass to stain proteoglycans (blue stain) produced by the chondrogenic cells (Fig. 9). Based on a visual comparison, there was no difference detected in differentiation between hMSCs cultured on T-175 Corning CellBIND Surface control flasks and HYPER*Stack* Vessels; however, no histological studies were performed.

The ability of the hMSCs cultured in Corning stemgro® hMSC Medium to differentiate into adipogenic and chondrogenic lineages, further demonstrates the capability of this system to support large scale expansion without the loss of cellular function.

Summary/Conclusion

Regenerative medicine, tissue engineering and bio-banking have created a demand for large scale production of hMSCs. Corning stemgro hMSC Medium in combination with Corning CellBIND Surface and HYPER technology have been successfully utilized as a method to scale-up hMSCs in serum-free conditions while still maintaining all characterizations/criteria of hMSCs.

- stemgro hMSC Medium enables serum-free proliferation of hMSC cultures on the Corning CellBIND Surface without the need for biological coatings.
- Successful expansion of hMSCs in stemgro hMSC Medium can be achieved while retaining the three main criteria used to define MSCs: proliferation, phenotypical characterization and multipotency.
- Corning hMSC Medium enables easy expansion into the HYPER*Stack* Cell Culture Vessel for large-scale expansions. We observed significantly higher rates of adipocyte differentiation of hMSCs harvested from the HYPER*Stack*-12 Cell Culture Vessel.

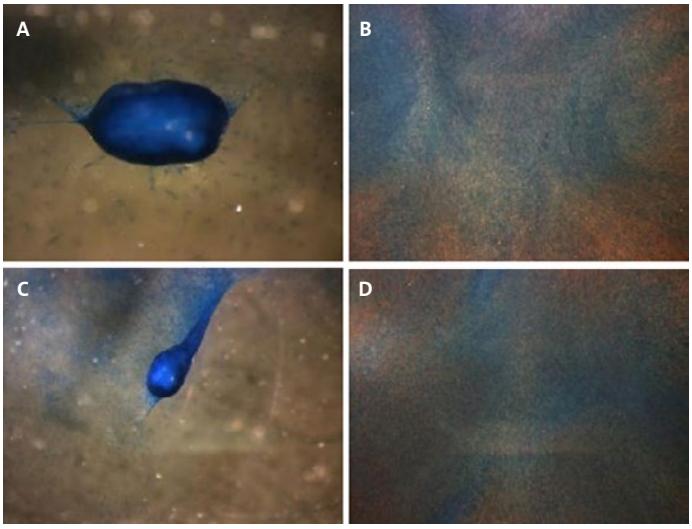

Figure 9. Chondrogenic assessment using Alcian blue stain. Representative example of chondrocyte differentiation from 4th expansion study, Alcian blue staining of proteoglycans (blue) synthesized by chondrocytes. Top: hMSCs harvested from Corning CellBIND Surface HYPER*Stack*-12 Vessel; (A) differentiated and (B) non-differentiated. Bottom: hMSCs harvested from T-175 Corning CellBIND Surface control flask; (C) differentiated and (D) non-differentiated.

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