Expansion of Human Mesenchymal Stem Cells Using Corning® HYPER Technology Cell Culture Vessels

CORNING

SnAPPshots

A brief technical report from the Corning Development Group

Ana Maria P. Pardo and Mark E. Rothenberg, Ph.D.
Corning Incorporated, Life Sciences
Kennebunk, ME 04043

Introduction

Mesenchymal stem cells (MSCs) are multipotent cells that can differentiate into a variety of cell types including osteoblasts, chondrocytes, and adipocytes when cultured under the correct conditions. A number of clinical trials with the application of autologous or allogenic human MSCs (hMSCs) are under way for a variety of conditions, such as Graft vs. Host disease, Crohn's disease, Acute Myocardial Infarction, and Type I Diabetes.[1,2] Thus the demand to define methods for scaling up hMSCs drives the requirement for robust production methodologies. Corning's HYPER (**H**igh **Y**ield **PER**formance) technology vessels are ideal products for the scale-up and recovery of large quantities of hMSCs.

The HYPER technology platform utilizes a gas permeable film to eliminate the requirement for an air gap normally found in T-flask and stacked chamber cell culture vessels. The HYPER vessels use up less space compared to traditional designs. Currently there are two product offerings utilizing the HYPER Technology. One is the HYPER*Flask*® Cell Culture Vessel, which has 1,720 cm^2 of cell growth surface area in a 10 layer vessel with the same spatial footprint as a T-175 flask (Figure 1A). The second is the HYPER*Stack*® Cell Culture Vessel (Figure 1B and C) which is designed for closed system applications and has approx-imately the same spatial footprint as commercially available 2, 10 and 40 layer Cell Culture Chambers.

Each HYPER*Stack* module is composed of 12 individual chambers (Figure 1B) which provide 6,000 cm^2 cell growth surface area. Other formats include the HYPER*Stack*-36 layer (18,000 cm^2, Figure 1C) and HYPER*Stack*-120 layer (60,000 cm^2) vessels. Both HYPER technology vessels are ideal for use in large scale production of cells, vaccines, and protein therapeutics.

The following study describes the use of the HYPER*Stack* vessels to scale-up hMSCs as compared to the more traditional use of stacked chamber vessels. hMSCs were thawed directly into HYPER*Flask* vessels. Cell number, viability and surface antigen profiles (positive antigens: CD105 and CD166 and negative antigens: CD14 and CD34) were assessed throughout the study.

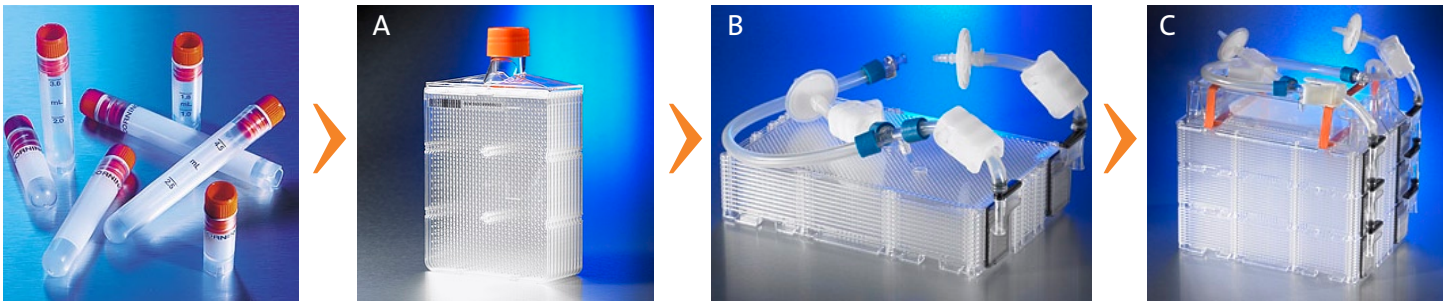

Figure 1. Representative Corning products used to scale-up hMSCs. Cells stored in Corning cryogenic vials (Cat. No. 430487) are thawed and sequentially passaged through the (A) Corning HYPER*Flask* vessel, (Cat. No. 10034) (B) HYPER*Stack*–12 Layer (Cat. No. 10012) and (C) HYPER*Stack*–36 Layer (Cat. No. 10036) vessels.

Table 1. Harvest volumes used in scaling-up hMSCs in vessel platforms. (SV = Stacked vessel)

	HYPER*Flask* (1720 cm²)		HYPER*Stack*–12 (6,000 cm²)		2 Layer SV (1,264 cm²)		HYPER*Stack*–36 (18,000 cm²)		10 Layer SV (6,320 cm²)	
	mL/cm²	mL	mL/cm²	mL	mL/cm²	mL	mL/cm²	mL	mL/cm²	mL
HBSS Rinse	0.03	50	0.03	200	0.02	30	0.03	600	0.02	150
Trypsin	0.03	50	0.03	200	0.02	30	0.03	600	0.02	150
Quench Media	0.03	50	0.03	200	0.02	30	0.03	600	0.02	150
HBSS Rinse	0.03	50	0.03	200	0.02	30	0.03	600	0.02	150
Media Rinse	0.03	50	0.03	200	0.02	30	0.03	600	0.02	150
Total Volume		200		800		120		2400		600
Cent. Tube		250 mL		2x 500 mL		250 mL		500 mL		3x 250 mL
Cent. Time (@ 600 x g)		10 min		15 min		10 min		15 min		10 min

Methods and Materials

Cell cultures: Bone derived human mesenchymal stem cells; hMSC's (Lonza, Cat. No. PT2501) were cultured in complete MSCGM™ medium (Lonza, Cat. No. PT3001).

The use of Poloxamer 188 in hMSC harvesting: hMSC's were seeded at a concentration of 5,000 cells/ cm² in Corning HYPER*Flask*® M (HF, Corning, Cat.No. 10034) and Corning CellBIND® Surface T75 (Corning, Cat. No. 3290) cell culture vessels and cultures grown to 80% confluence (5 days) in a humidified incubator set to 37°C and 5% CO_2. A full medium change was done 24 hours prior to harvest. On the day of harvest medium was aspirated from the vessel followed by a gentle rinse using 1x HBSS (Corning cellgro®, Cat. No. 21-023-CM). Cells were harvested using trypsin/EDTA (Corning cellgro, Cat. No. 25-052-CV) with and without 0.1% Poloxamer 188, (Corning cellgro, Cat. No. 13-901-CI). To harvest, the harvest solution was gently distributed throughout the vessels for 1 minute then incubated for 4 minutes at 37°C. Once most of the cells were released from the growth surface, the trypsin/Polaxamer solution was collected into an equal volume of growth medium. The empty vessel was incubated at room temperature for approximately 3 minutes to allow remaining cells to detach from the growth surface. Cell exposure to trypsin was always kept to under 10 minutes. Cells were collected from the vessels with an HBSS rinse and pooled with the initial collection. A second vessel rinse was done using complete growth medium. Cell concentration and viability was determined using a BioProfile® FLEX instrument (Nova Biomedical). The cell suspension was concentrated by centrifugation at 600 x g for 5 to 10 minutes and re-suspended in fresh media. Cell concentration and viability were re-assessed using the Bioprofile FLEX instrument. Cells from each harvest were re-seeded in HYPER*Flask* and T75 vessels to repeat the harvest and recovery testing a total of 6 independent times.

Thaw and Maintenance: Frozen hMSC stocks were thawed and prepared for culture following Lonza's Instruction for Use Manual. Thawed cells were seeded into a Corning HYPER*Flask* M Cell Culture Vessel (HF, Corning Cat. No. 10034) at a concentration on 5,000 cells/ cm² in 0.326 mL/ cm² and designated passage #0. Cells were cultured to 90% confluence (6 or 7 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 medium.

Subculture and Scale-up: On the day of harvest (90% confluent cultures) medium was aspirated from the vessel and harvested as described above. Once collected, cell concentration and viability were assessed using the BioProfile FLEX instrument. The cell suspension was concentrated by centrifugation at 600 x g for 10 to 15 minutes (time based upon cell volume of cell concentration, see Table 1) and re-suspended in fresh medium. hMSC concentration and viablity was re-assesed using the BioProfile FLEX instrument, cells were then seeded into a Corning® HYPER*Stack*®-12 Cell Culture Vessel (HS-12, Corning Cat. No. 10012) and a 2 layer stacked cell culture vessel (SV-2, Nunc Cat. No. 167695) both at a concentration of 5,000 cells/cm² in 0.217 mL/cm² complete culture medium. Cultures were designated passage #1, maintained and subcultured as described above. A small sample of cell suspension (1-5 mL) was saved after each harvest for phenotypic analysis.

HYPERStack-36 Scale-up: hMSCs harvested from the HYPER*Stack*-12 vessel and the 2 layer stacked cell culture vessel were used to seed a Corning HYPER*Stack*-36 Cell Culture Vessel (HS-36, Corning Cat. No. 10036) and a 10 layer stacked cell culture vessel (SV-10, Nunc Cat No. 164327) as described above. These cultures were designated passage #2 and maintained for 5 days without a medium change before harvest. This procedure was repeated twice with cultures from frozen stocks and once using cells harvested from the passage #2 cultures.

hMSC Morphologic and Proliferation Assessment: To monitor the general health of the hMSC cultures, population doublings and morphologic changes were assessed via microscopic examination using an inverted Olympus microscope. Characteristics of the cultures were analyzed after each harvest. Population doublings were assessed by using the equation $n = 3.32\ (logN\text{-}logX)$, where n = population doubling, N = Total # of cells harvested and X = Initial # of cells plated.

Surface Marker Analysis: To assess hMSC surface markers, harvested cells 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 all residual culture medium. Cells were fixed by re-suspending 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-155-250mL) and stored at 4°C until analysis. On the day of analysis, the samples were pelleted by centrifugation (600 x g for 5 min) to remove fixing solution, re-suspended in blocking buffer (0.5% BSA in staining buffer) to a density of approximately 10 x10^6 cells/mL and incubated for 15 minutes at room temperature.

After blocking, 100 µL of each sample (~1.0 x10^6 cells/mL) was transferred into a 2 mL sample tube and marked as follows: no stain control, IgG1 isotype control, CD105, CD166, CD14 and CD34 (R&D systems, Cat. No.'s IC002P, FAB10971P, FAB6561P, FAB3832P and FAB7227P, respectively).

10 µL of corresponding conjugated antibody or isotype control was then added to each sample tube, mixed well and incubated for 30 to 45 minutes at 4°C. After incubation, cells were washed and pelleted by centrifugation 2 times using SB to remove unbound antibody (4 minutes at 300 x g). Cells were re-suspended in 200 to 500 µL of SB for flow cytometry analysis using MACSQuant® Analyzer instrument (Miltenyi Biotec).

Results

Cell Harvest and Recovery: To increase yields of hMSC's 0.1% Poloxamer 188 was used in conjunction with trypsin. Pluronic F68, also known as Poloxamer 188, is a tri-block co-polymer that is a synthetic nonionic surfactant used in a variety of cell culture applications including bioreactors to reduce cell damage and increase cell yield[3]. Figure 2 shows how the use of 0.1% Poloxamer with trypsin increased recovery of hMSCs post harvest and reduced loss due to centrifugation compared to those harvested where Poloxamer was not utilized.

The hMSCs were seeded from thaw into a HYPER*Flask*® vessel and the cell number was scaled-up by sequential passaging through the HYPER*Stack*®-12 followed by the HYPER*Stack*-36 vessel.

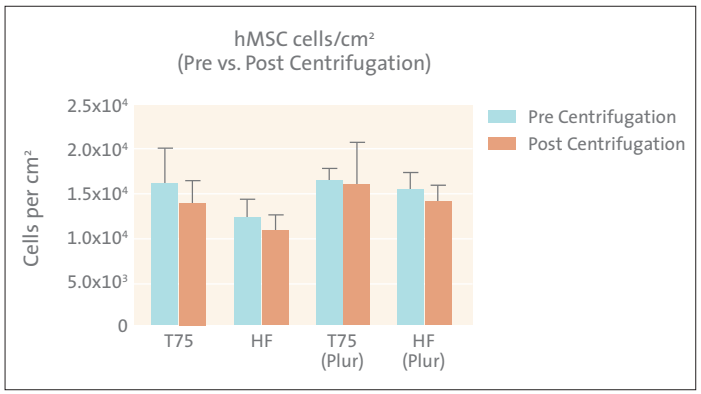

Figure 2. hMSC harvest using Trypsin/EDTA with 0.1% Poloxamer (Plur). Addition of Poloxamer to trypsin solutions facilitates full recovery of cells from HYPER*Flask* cell culture vessel and decreases loss associated with centrifugation (n = 6 from 5 independent studies).

The goal of the following analysis was to compare yield between the two vessel types used in this study. The average yields from sub-confluent cultures (5 days in culture without media change) was 154 x 10^6 cells collected from HYPER-*Stack*-36 vessels compared to the 55 x 10^6 cells harvested from the 10 layer stacked cell culture vessels (Figure 3). The cell number per growth area (cm^2) showed no statistical difference of cell growth between the two vessel types (Figure 4).

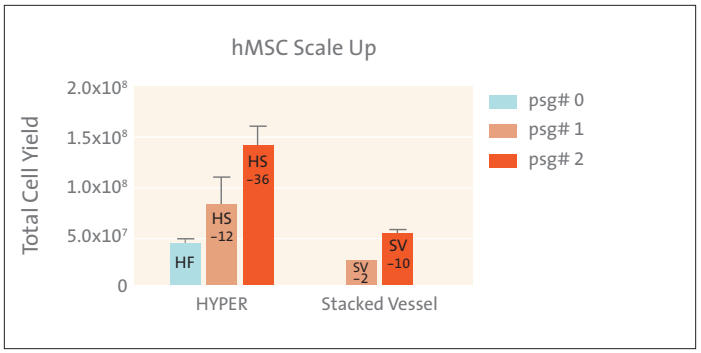

Figure 3. Comparison of hMSC total yield from the various vessel types used in the study at various passage numbers (psg#). (HF) HYPER*Flask* (HF), HYPER*Stack* 12 (HS–12) or HYPER*Stack* 36 (HS-36) and 2 and 10 layer Stacked Culture Vessels (SV–2 and –10). Data from n = 3 independent studies.

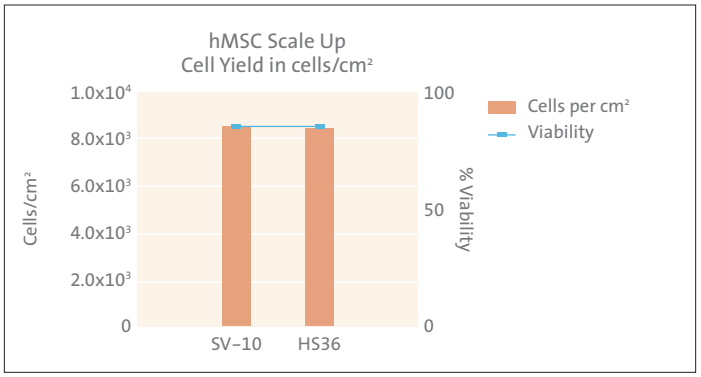

Figure 4. hMSC yields in cells/cm^2. hMSC's were cultured for 5 days and harvested prior to reaching full confluency. No significant difference in yields or viability between HYPER*Stack*–36 Cell Culture Vessel or the stacked 10 layer vessel. (n = 4, ANOVA value $p < 0.001$)

Cell Behavior: Human MSC cultures from both vessel types were evaluated after each harvest to monitor the viability and surface markers of the harvested cells. Morphologic examination (Figure 5) showed no observable differences between cells cultured in HYPER vessels compared to stacked vessels. Population doublings were calculated for every harvest using the equation $n = 3.32\ (logN\text{-}logX)$. The results demonstrate similar population doublings between the two types of culture vessels throughout the length of the study (Figure 6).

To evaluate the phenotype of the hMSCs over time, cell samples from each harvest were collected and analyzed for positive and negative surface marker expression with flow cytometry. CD105 and CD166 markers were used as positive expression markers while CD14 and CD34 were used as negative expression markers. These markers were chosen based on recommendation from Lonza.[3,4] Flow cytometric analysis demonstrated similar expression levels of the various surface markers between all cell culture vessels throughout the length of the culture study with > 99% positive expression for CD105 and CD166 and < 5% expression for CD14 and CD34. (Figure 7).

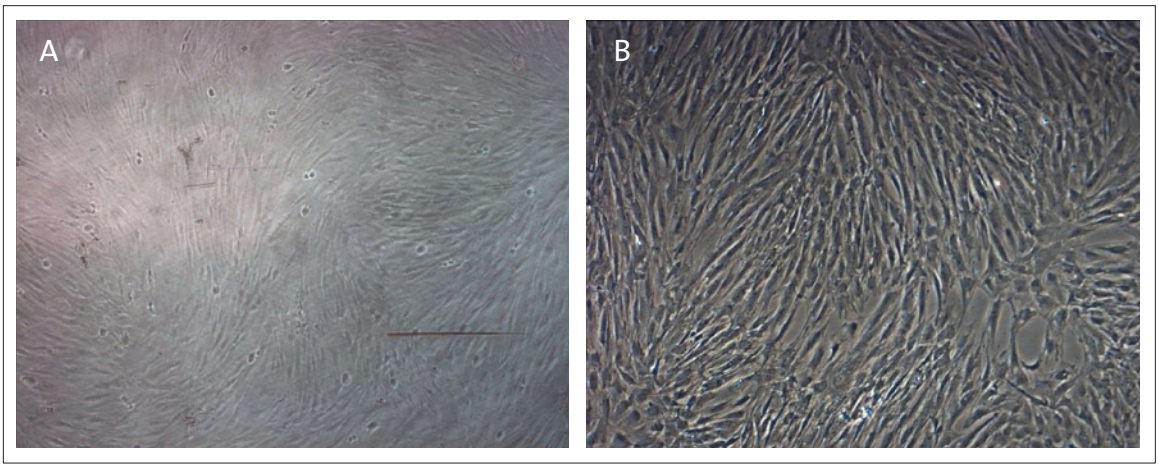

Figure 5. Morphological comparison of hMSC culture. Representative micrographs of confluent hMSC cultures on; A) Corning HYPER*Flask* Cell Culture Vessel (passage#0), B) 2 layer stacked culture vessel (passage #1) after 144 hours in culture. (magnification = 4x using inverted Olympus microscope).

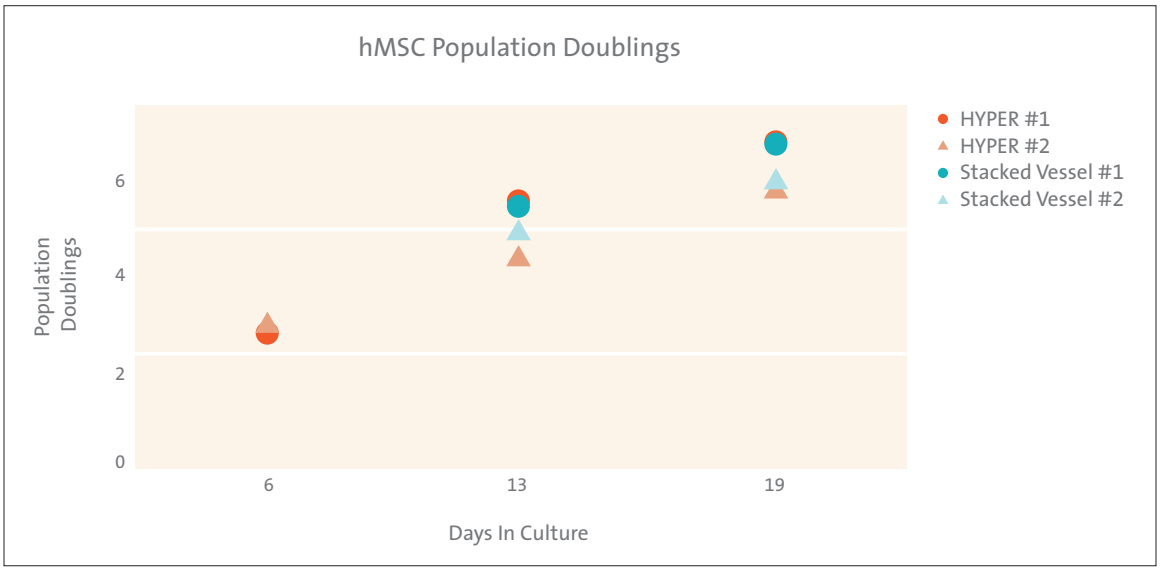

Figure 6. Cumulative hMSC population doublings for both vessel conditions throughout length of study. No significant differences in doubling times were observed comparing the two vessel types. Representative samples from 2 independent studies (#1 and #2) are shown (Day 6 data from HYPER*Flask* vessels. Day 13 data from HYPER*Stack*–12 and 2 layer stacked vessels. Day 19 data from HYPER*Stack*–36 and 10 layer stacked vessels).

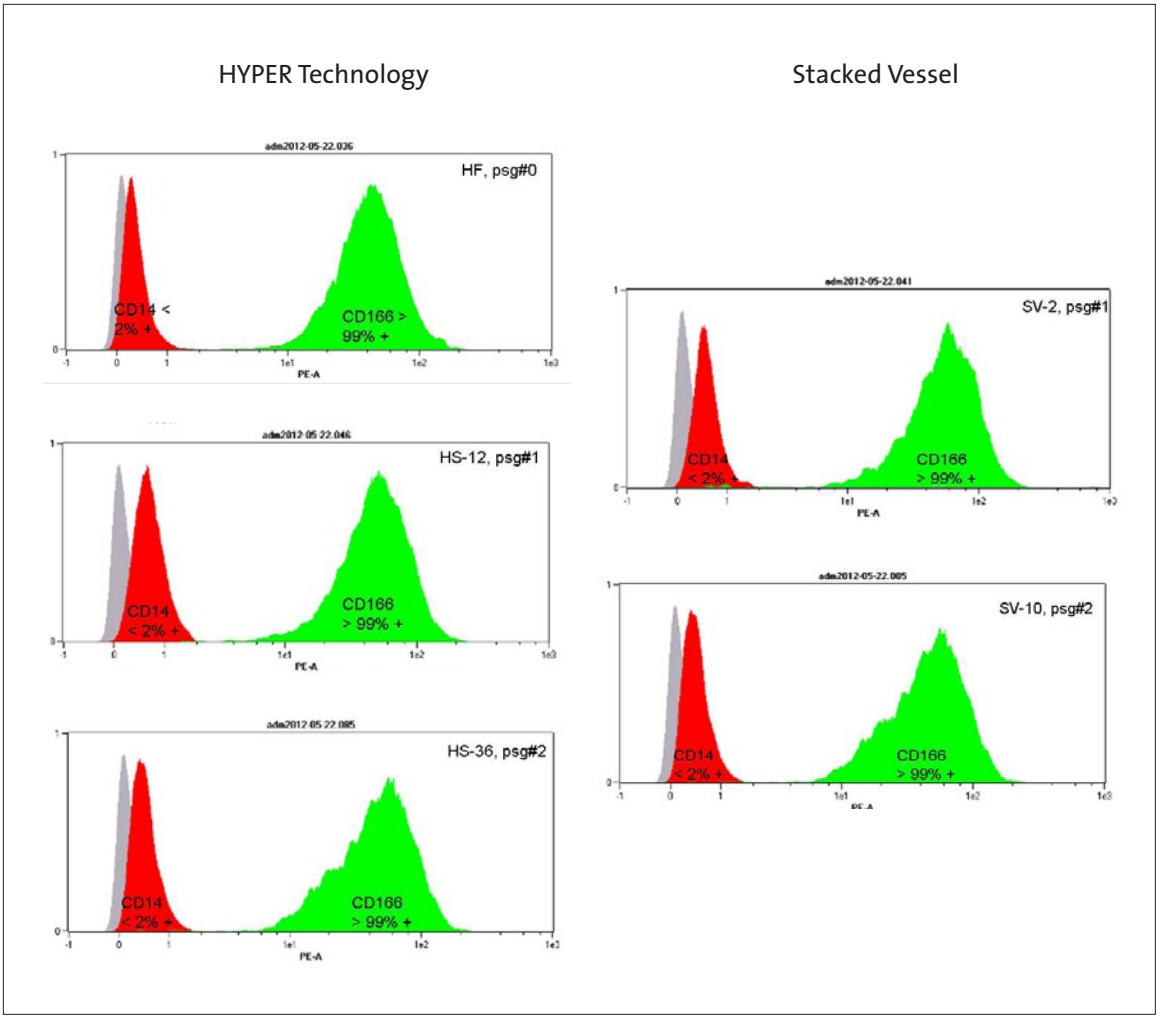

Figure 7. Representative flow cytometry data. Expression of CD166 (+green) and CD14 (-red) surface markers was observed for all the HYPER vessels and stacked vessel cultures analyzed. The gray peak is indicative of the isotype control. (Study repeated n = 3 independent times).

Summary/Conclusion

Stem cell use in cell therapy and tissue engineering applications is growing, and so is the requirement for cell culture technologies to allow for this growth. The Corning® HYPER Technology Platform vessels which include the HYPER*Flask*® and the HYPER*Stack*® Cell Culture Vessels, can be successfully utilized to scale-up hMSCs with expected viability, surface markers and proliferation rates.

- hMSC morphology and proliferation rates for cells cultured in HYPER*Flask* and HYPER*Stack* cell culture vessels were equivalent to stacked vessels.
- Corning HYPER Technology vessels are an excellent option for scaling-up large quantities of hMSCs for use in a number of cell therapeutic applications.

References

1. P-HG Chao, W. Grayson, and G. Vunjak-Novakovic, J. Orthop. Sci. 12 (4), 398-404 (2007).
2. M. von Bonin et al., Bone Marrow Transplant 43 (3), 245-251 (2009).
3. E.M. Horwitz, M. Andreef and Francesco Frassoni, Biology of Blood and Marrow Transplantation 12, 59-57 (2007).
4. S. K. Both, et al., A Rapid and Efficient Method for Expansion of Human Mesenchymal Stem Cells, Advances in Tissue Engineering: Volume 2. 235-241 (2010).

For additional product or technical information, please visit **www.corning.com/lifesciences** or call 1.800.492.1110. Outside the United States, please call 978.442.2200.

Corning Incorporated
Life Sciences
836 North St.
Building 300, Suite 3401
Tewksbury, MA 01876
t 800.492.1110
t 978.442.2200
f 978.442.2476

www.corning.com/lifesciences

Worldwide Support Offices

ASIA/PACIFIC

Australia/New Zealand
t 0402-794-347

China
t 86 21 2215 2888
f 86 21 6215 2988

India
t 91 124 4604000
f 91 124 4604099

Japan
t 81 3-3586 1996
f 81 3-3586 1291

Korea
t 82 2-796-9500
f 82 2-796-9300

Singapore
t 65 6733-6511
f 65 6861-2913

Taiwan
t 886 2-2716-0338
f 886 2-2516-7500

EUROPE

France
t 0800 916 882
f 0800 918 636

Germany
t 0800 101 1153
f 0800 101 2427

The Netherlands
t 31 20 655 79 28
f 31 20 659 76 73

United Kingdom
t 0800 376 8660
f 0800 279 1117

All Other European Countries
t 31 (0) 20 659 60 51
f 31 (0) 20 659 76 73

LATIN AMERICA

Brasil
t (55-11) 3089-7419
f (55-11) 3167-0700

Mexico
t (52-81) 8158-8400
f (52-81) 8313-8589

Distributor
Corning International K. K.
Life Sciences
Akasaka Intercity 6th floor
1–11–44 Akasaka, Minato-ku
Tokyo 107–0052, Japan
t 81 3–3586 1996
f 81 3–3586 1291
e CLSJP@corning.com

The Corning Family of Brands

CORNING COSTAR cellgro® PYREX® AXYGEN® GOSSELIN™

Corning Incorporated, One Riverfront Plaza, Corning, NY 14831–0001