

A Xeno-Free Culture System for hMSC from Various Sources Suitable for Initial Isolation and Expansion Toward Clinical Applications

Mira Genser-Nir, Sharon Daniliuc, Marina Tevovsky, David Fiorentini.
Biological Industries, Beit Haemek, Israel.

Abstract

Human mesenchymal stem cells (hMSC) are multipotent adult stem cells present in a variety of tissue niches in the human body. hMSC have advantages over other stem cell types due to the broad variety of their tissue sources, since they are immuno-privileged, and for their ability to specifically migrate to tumors and wounds in vivo. Due to these traits hMSC have become desirable tools in tissue engineering and cell therapy. In most clinical applications hMSC are expanded in vitro before use. The quality of the culture medium and its performance are particularly crucial with regard to therapeutic applications, since hMSC properties can be significantly affected by medium components and culture conditions.

To date there is no efficient xeno-free (XF) medium for the initial isolation of hMSC from various tissues. In addition, most of the common culture media for growth and expansion of hMSC, as well as auxiliary solutions (for attachment, dissociation, and cryopreservation), are typically supplemented with serum or other xenogenic compounds. A defined serum-free (SF), XF culture system optimized for hMSC isolation and expansion would greatly facilitate the development of robust, clinically acceptable culture process for reproducibly generating quality-assured cells.

The present study evaluated a novel XF culture system, comprising MSC NutriStem® XF culture medium and all the required auxiliary solutions for the attachment, dissociation, and cryopreservation of the cells. The system was evaluated for initial isolation of hMSC from various sources, and for long-term culturing under SF, XF culture conditions suited for clinical applications.

Results show that the XF culture system for hMSC efficiently supports initial isolation and optimal expansion of hMSC from various sources, while maintaining MSC features: typical fibroblast-like cell morphology, phenotypic surface marker profile, differentiation capacity, self-renewal potential, and genetic stability.

Abbreviations
FBS Foetal Bovine Serum
hMSC Human Mesenchymal Stem Cells
hMSC-AT Adipose Tissue derived hMSC
hMSC-BM Bone Marrow derived hMSC

hMSC-PL Placenta derived hMSC
hMSC-WJ Wharton's Jelly derived hMSC
SF Serum Free
XF Xeno Free

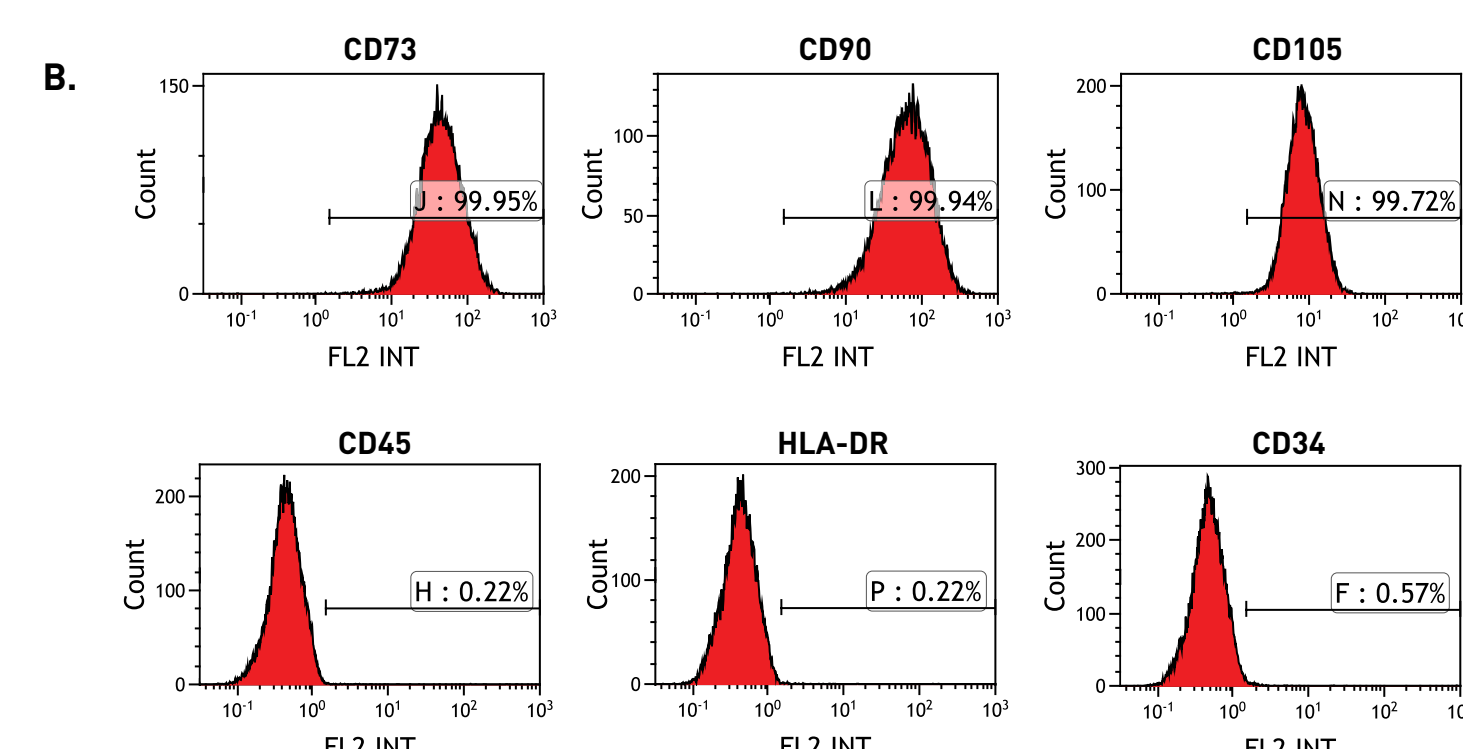
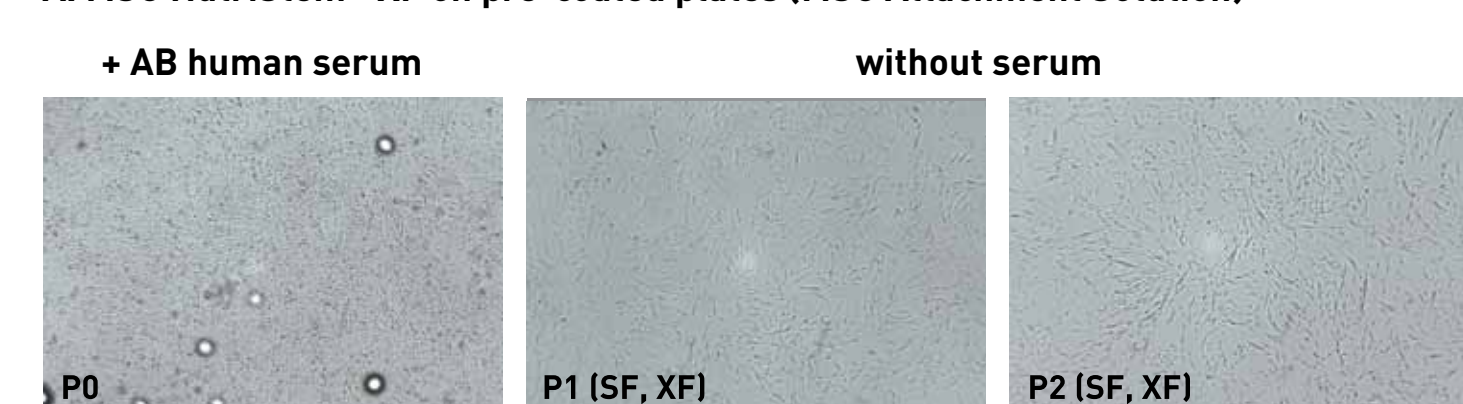
Results

I. INITIAL ISOLATION

hMSC-AT

Figure 1: Evaluation of hMSC-AT isolation using MSC NutriStem® XF

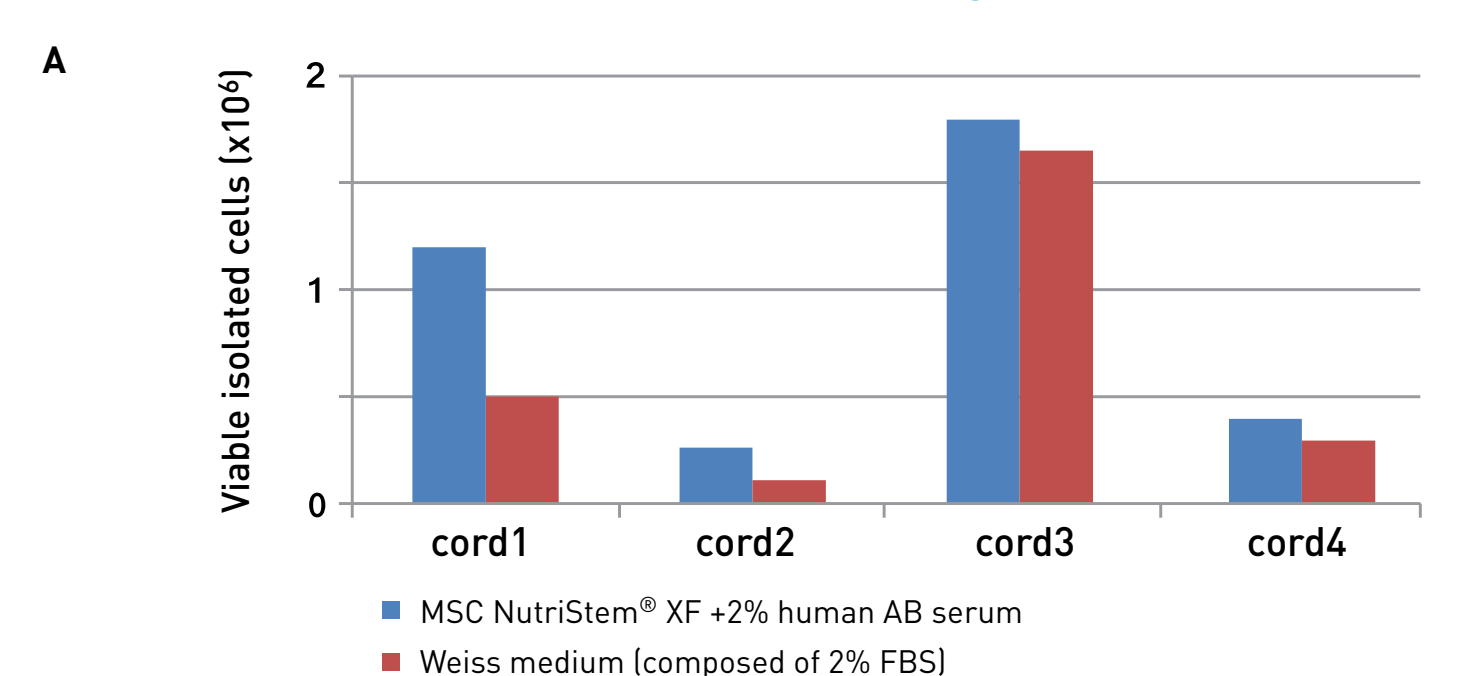
A. MSC NutriStem® XF on pre-coated plates (MSC Attachment Solution)



Adipose tissue-derived cells were seeded in MSC NutriStem® XF supplemented with 2% human AB+ serum on pre-coated plates with MSC Attachment Solution for the initial isolation and expansion of hMSC-AT (P0). The cells were cultured to 70-80% confluence before being sub-cultured. Further passages (P1-2) were done under SF, XF culture conditions, utilizing MSC NutriStem® XF culture medium on pre-coated dish. **A.** Representative images taken 4 days post initial seeding (P0) and 3 days post P1 and P2. **B.** Immunophenotyping results of hMSC-AT at passage 2 using FACS analysis. **Successful isolation of hMSC-AT that maintains a classical profile of MSC markers was achieved under XF conditions, utilizing MSC NutriStem® XF medium.**

hMSC-WJ

Figure 2: Comparison of hMSC-WJ isolation utilizing MSC NutriStem® XF vs. FBS-containing medium



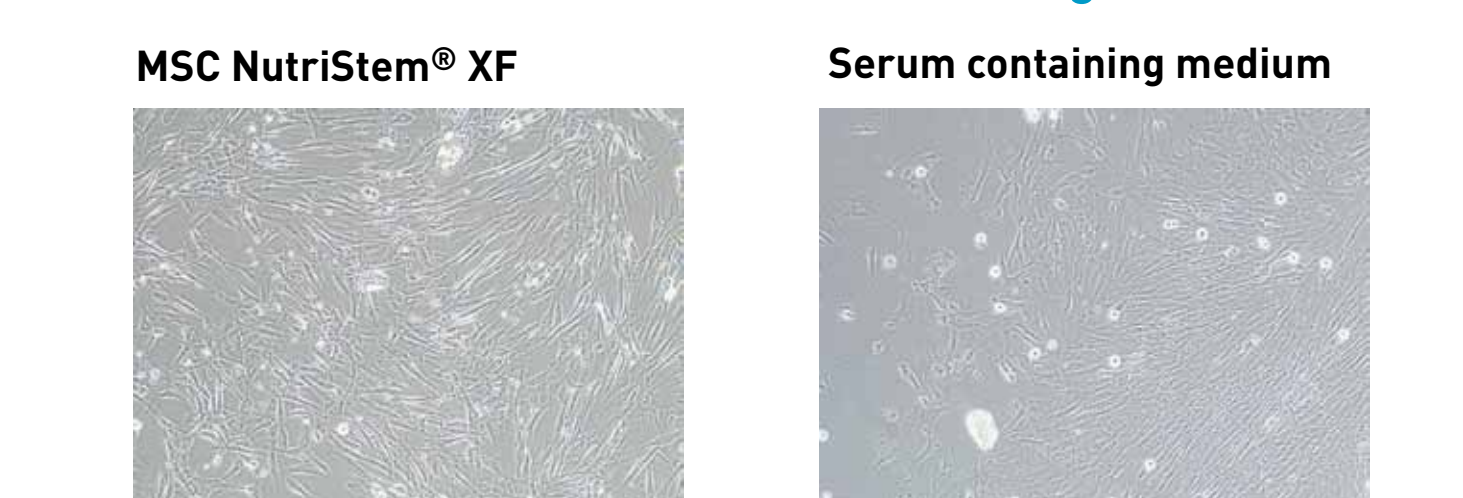
B.

	Day 2 post initial isolation		Day 7 post initial isolation	
	Representative images	Live	Live	Dead
MSC NutriStem® XF (supplemented with 2% human AB+ serum)		395,000	15,000	
Weiss medium (composed of 2% FBS)		295,000	75,000	

hMSC were initially isolated from 4 independent human cords utilizing XF medium (MSC NutriStem® XF supplemented with 2% human AB+ serum on pre-coated plates with MSC Attachment Solution) in comparison to serum-containing medium. **A.** Comparing the amount of viable cells - passage 0. Cell count was measured by trypan blue exclusion assay. **B.** Representative images (x40) of cord 4 taken on Day 2 post initial isolation in each medium, and cell count results of Day 7 post initial isolation, respectively. **Initial isolation of hMSC-WJ under XF culture system is superior.**

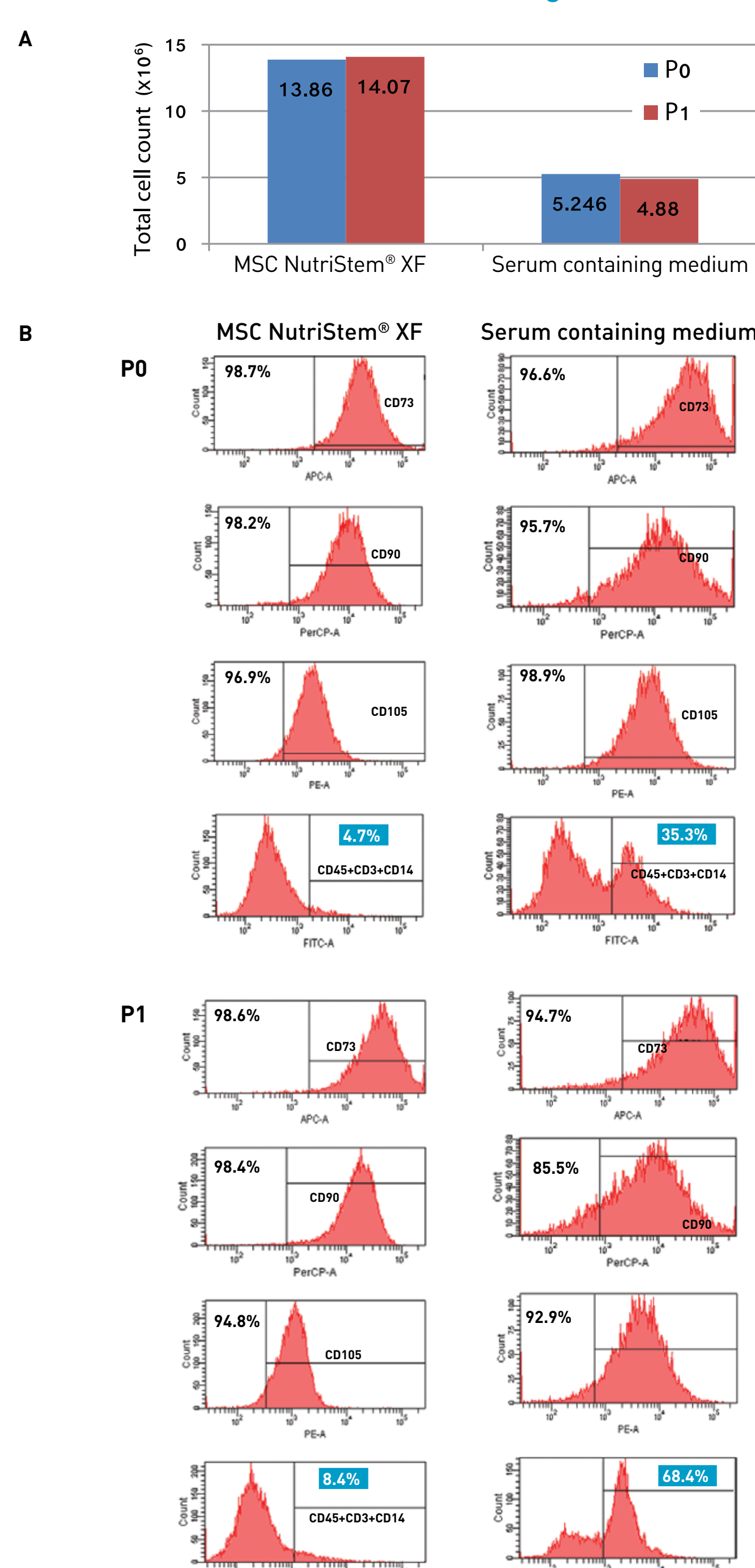
hMSC-PL

Figure 3: Isolation of hMSC-PL using SF, XF culture medium (MSC NutriStem® XF) and serum-containing medium



hMSC were isolated from frozen crude placenta under SF, XF culture conditions [MSC NutriStem® XF on pre-coated plates with MSC Attachment Solution, w/o supplementation of human AB+ serum] and in FBS containing medium. Representative images (x40) taken 11 days post initial isolation (P0). **Higher confluence is observed utilizing MSC NutriStem® XF w/o the requirement of human AB+ serum supplementation.**

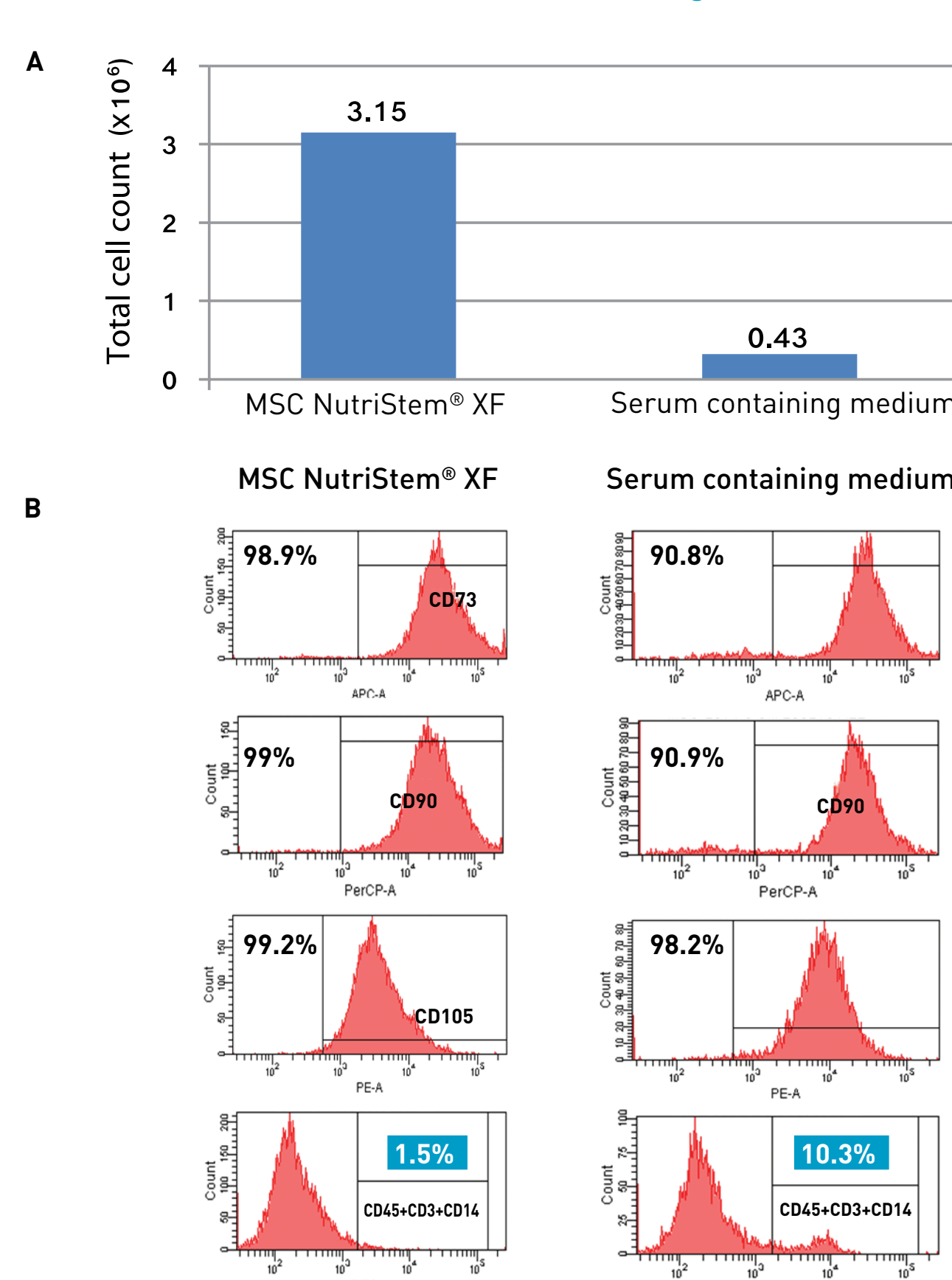
Figure 4: Evaluation of hMSC-PL isolation using MSC NutriStem® XF vs. FBS containing medium



Comparison of hMSC-PL isolation from crude placenta 17 days post initial seeding (P0) and 7 days post P0 (P1) in each medium. **A.** Quantity of viable cells, measured by trypan blue exclusion assay. **B.** Immunophenotype results using FACS analysis. **Initial isolation of hMSC-PL under SF, XF culture system using MSC NutriStem® XF (w/o supplementation of human AB+ serum) is superior; achieves a higher number of purer and viable cells that maintain a normal profile of MSC markers. Further expansion in MSC NutriStem® XF medium enhanced the advantages over serum-containing medium, leading to higher level of MSC purity (91.6% and 31.6%, respectively, emphasize in blue).**

hMSC-BM

Figure 5: Evaluation of hMSC-BM isolation using MSC NutriStem® XF vs. FBS containing medium



Comparison of hMSC-BM isolation from fresh BM utilizing MSC NutriStem® XF and serum-containing medium (11-day assay). **A.** Cell count was measured by trypan blue exclusion assay. **B.** Immunophenotype using FACS analysis. **Initial isolation of hMSC-BM using MSC NutriStem® XF is superior; achieves MSC populations with a higher number of viable cells and higher levels of purity (98.5% and 89.7% respectively, emphasize in blue) that maintain a classical profile of MSC markers.**

Materials and Methods

Cells

hMSC (passage 0-5) from a variety of sources: AT, WJ, PL and BM were used in this study.

Initial isolation of hMSC

hMSC AT, WJ, and PL were isolated by enzymatic digestion followed by centrifugal separation to isolate the stromal/vascular cells. The pellet was re-suspended with MSC NutriStem® XF supplemented with 2% human AB+ serum (hMSC-AT and WJ) or w/o the addition of serum (hMSC-PL), and cultured on pre-coated plates with MSC Attachment Solution.

hMSC-BM mononuclear cells were collected from a bone marrow aspirate by density gradient centrifugation. The cells were washed and cultured on pre-coated plates (with MSC Attachment Solution) using MSC NutriStem® XF and serum-containing medium. The marrow stromal cells were selected by plastic adherence and were allowed to expand until reaching sub-confluence.

SF, XF culture system

hMSC were cultured in a SF, XF expansion medium (MSC NutriStem® XF, BI) on pre-coated dishes (MSC Attachment Solution, BI) or as indicated. Cells were seeded at concentrations of 5000-6000 viable cells/cm² and harvested using either MSC Dissociation Solution (BI) or Recombinant Trypsin Solution (BI).

Medium performance evaluation

Medium performance was evaluated by viable cell count, proliferation rate, cell morphology, multilineage differentiation potential into adipocytes, osteocytes, and chondrocytes, self-renewal potential, cell immunophenotype, and karyotype analysis.

Differentiation

hMSC expanded for 3-5 passages in MSC NutriStem® XF were tested for multilineage differentiation potential (into adipocytes, osteocytes, and chondrocytes) using differentiation media. Cells were fixed and stained with Oil Red O, Alizarin Red, and Alcian Blue, respectively.

CFU-F

hMSC were seeded at low densities (10, 50, and 100 cells/cm²) in MSC NutriStem® XF on pre-coated dishes (MSC Attachment Solution, BI) or as indicated, and cultured for 14 days following staining with 0.5% Crystal violet.

Flow Cytometry

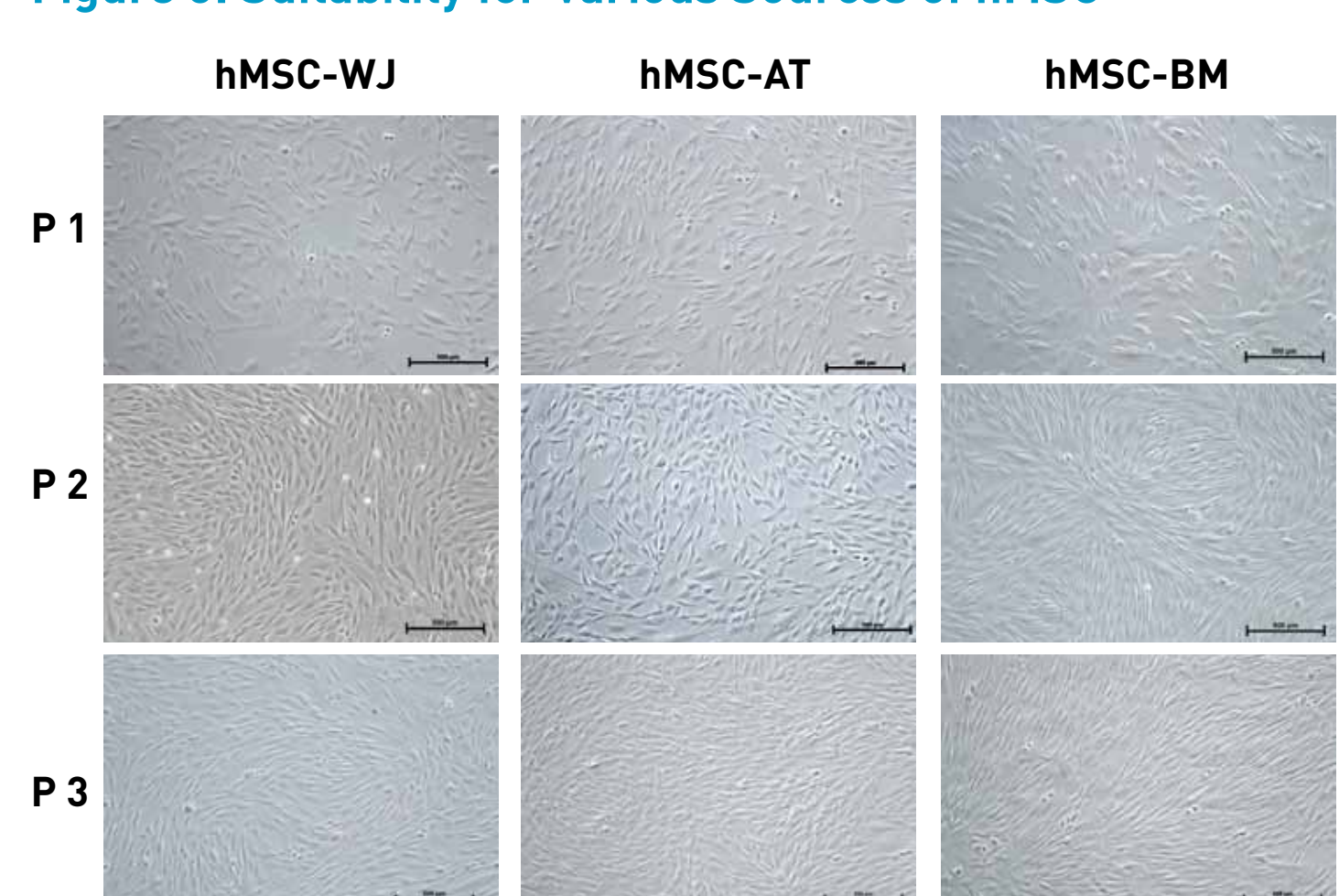
hMSC were cultured for 2-5 passages in MSC NutriStem® XF followed by MSC identification by flow cytometry using positive and negative surface markers.

Karyotype

Genomic stability of hMSC was tested by G-banding karyotyping analysis.

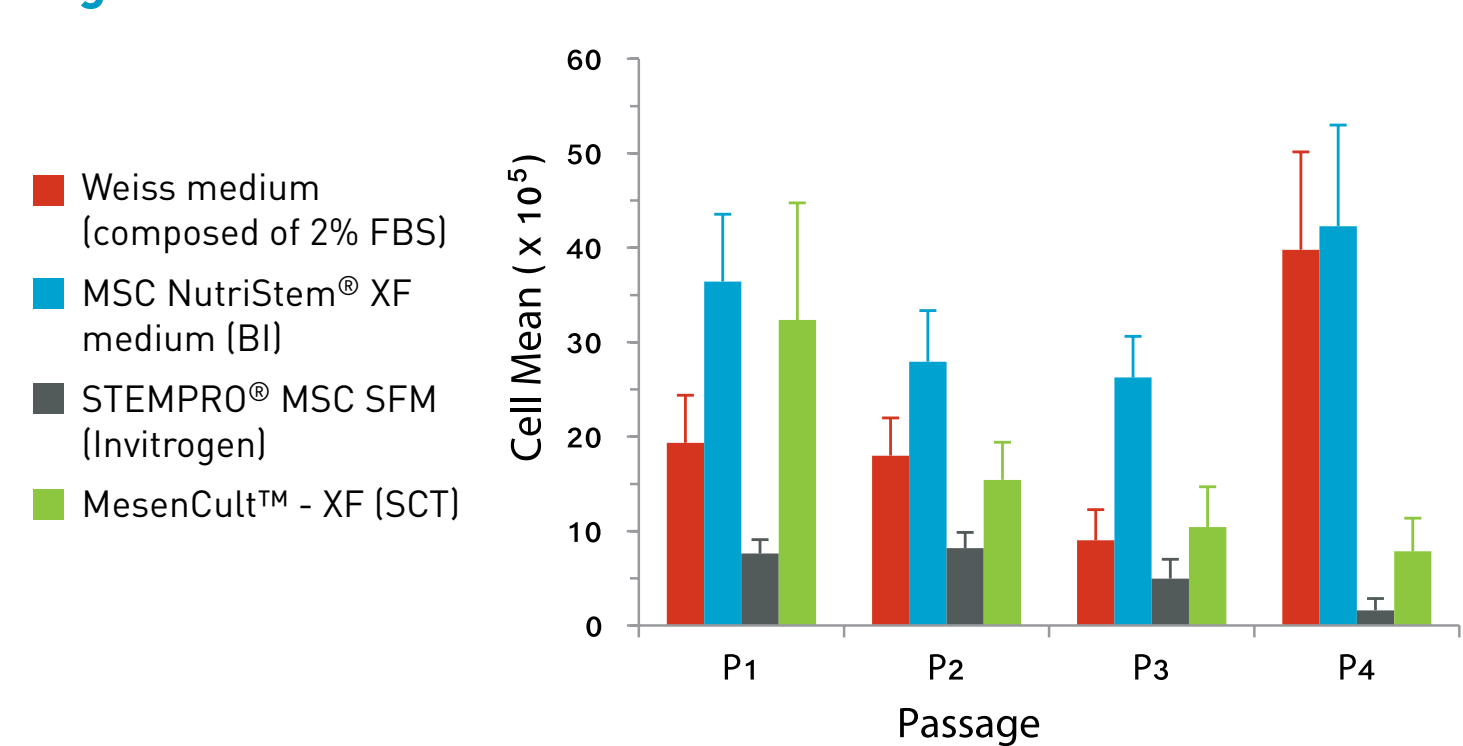
II. EXPANSION

Figure 6: Suitability for Various Sources of hMSC



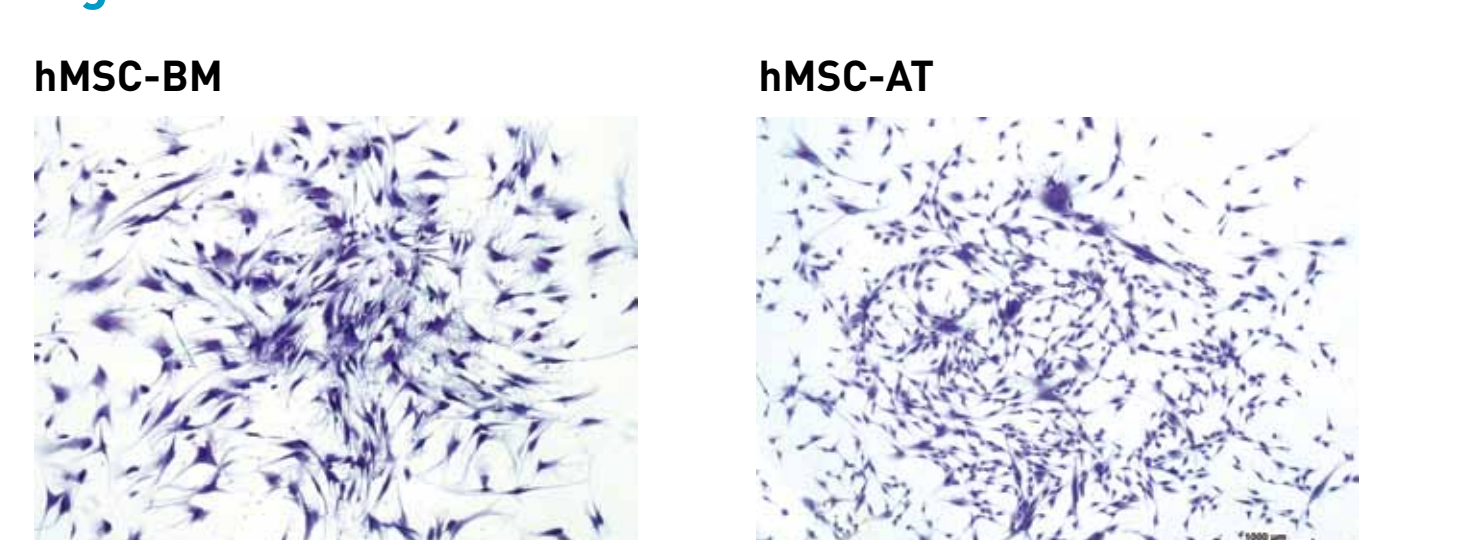
hMSC derived from a variety of sources: WJ, AT, and BM cultured for 3 passages in the XF culture system [MSC NutriStem® XF, MSC Attachment Solution, MSC Dissociation Solution]. Representative images taken on Day 3 of culture (x100). **MSC NutriStem® XF promotes proliferation of hMSC from a variety of sources while maintaining their fibroblast-like morphology.**

Figure 7: Proliferation



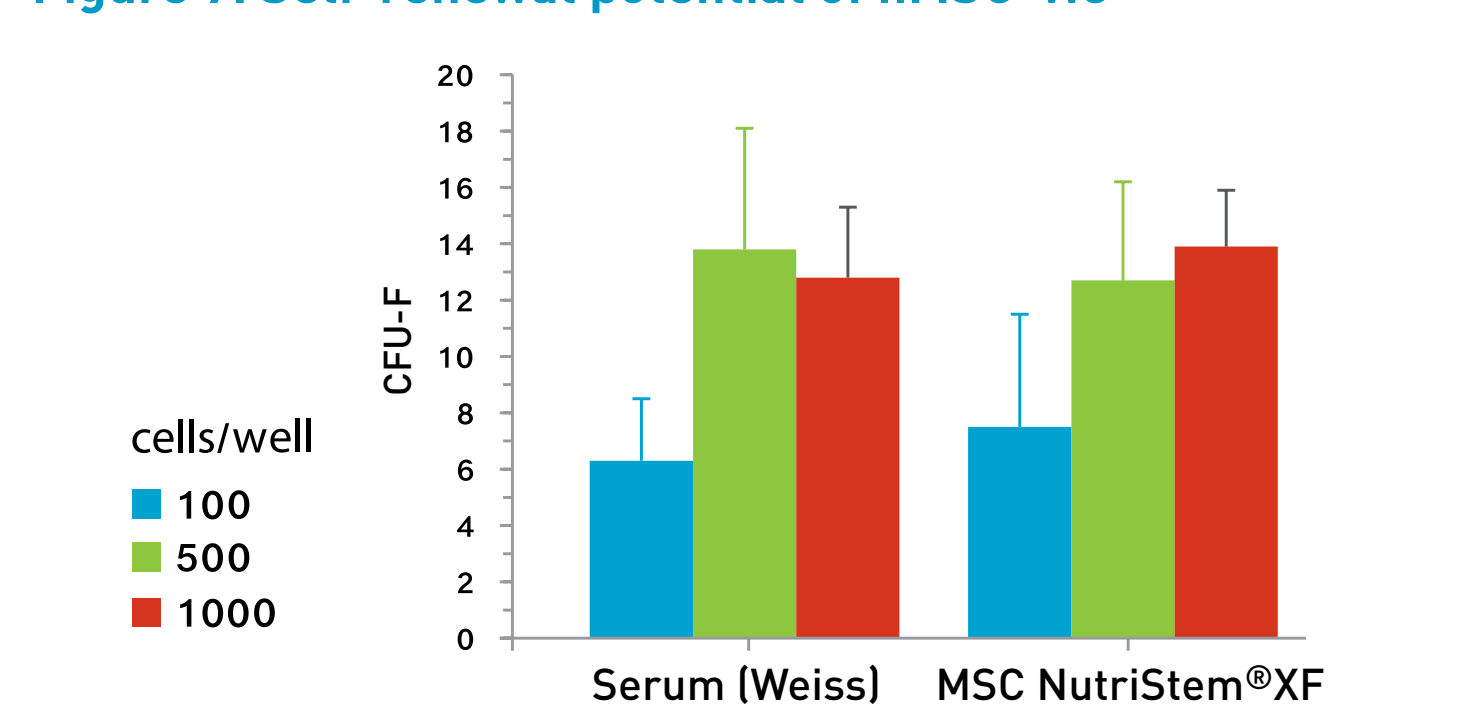
hMSC-WJ from nine different donors expanded for 4 passages in MSC NutriStem® XF in comparison to commercial SF media and commercial serum-containing medium. Cell proliferation was assessed by cell count using a trypan blue exclusion assay. **MSC NutriStem® XF produces the best overall expansion of hMSC-WJ.**

Figure 8: Self-renewal Potential



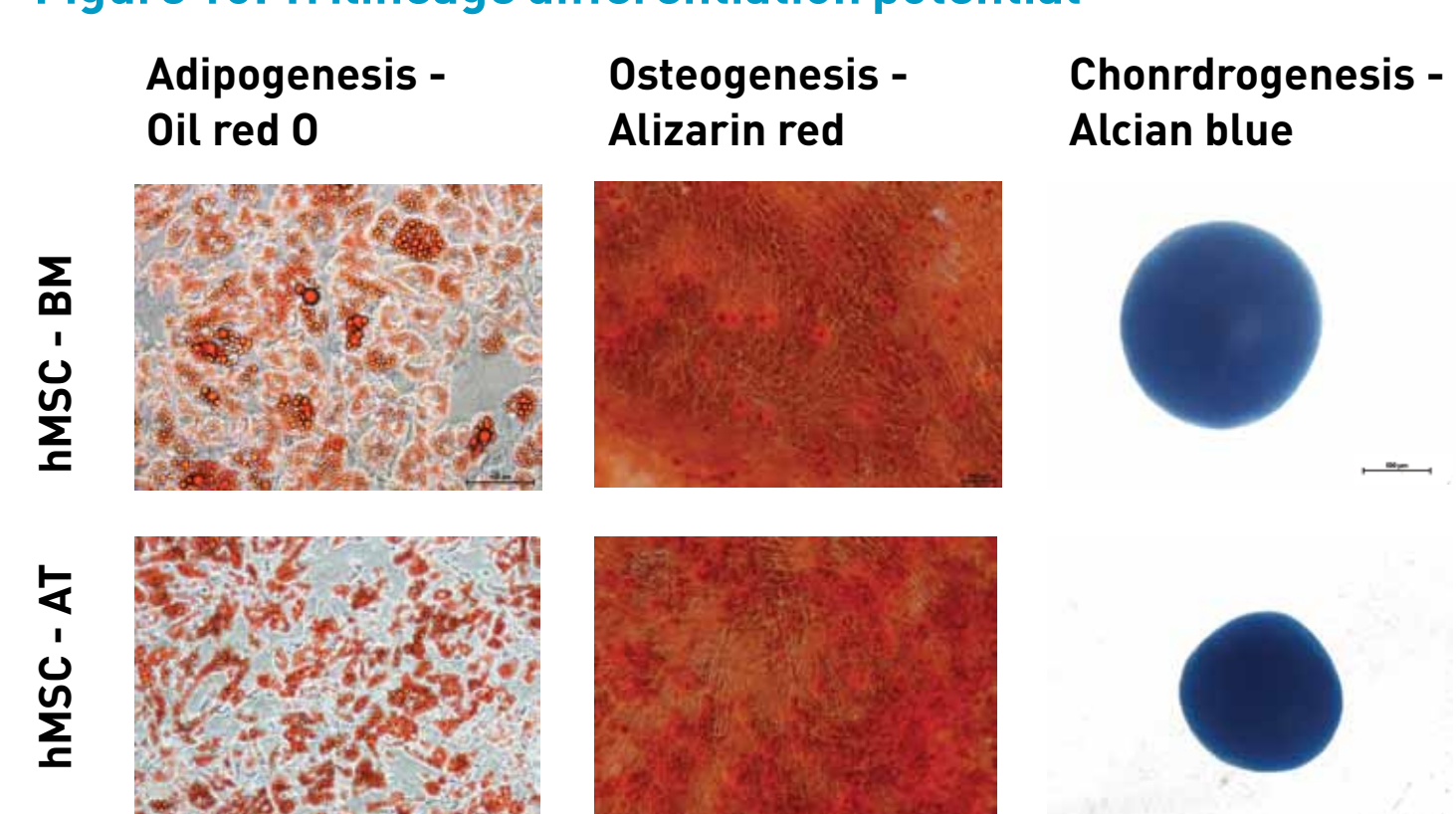
hMSC-BM and AT expanded in MSC NutriStem® XF for 3-5 passages prior to 14 days of CFU-F assay. Representative images of colonies stained with 0.5% crystal violet (x100). **hMSC cultured in MSC NutriStem® XF maintain their self-renewal potential.**

Figure 9: Self-renewal potential of hMSC-WJ



CFU-F assay of hMSC-WJ expanded for 5 passages in MSC NutriStem® XF and Weiss medium (2% FBS) in 3 different seeding concentrations. **hMSC cultured in MSC NutriStem® XF maintain their self-renewal potential.**

Figure 10: Trilineage differentiation potential



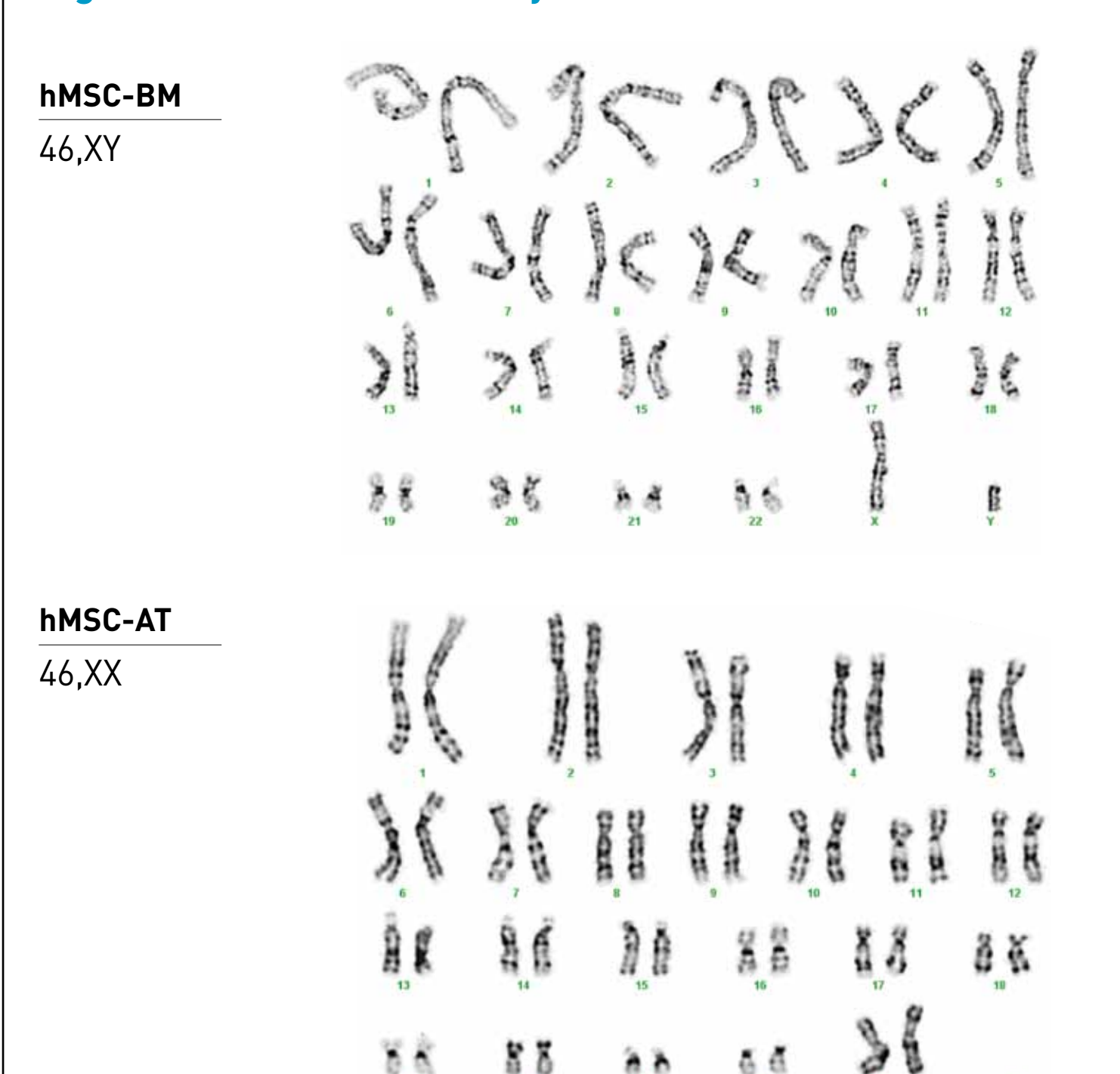
hMSC-BM and hMSC-AT expanded in MSC NutriStem® XF for 3-5 passages prior to trilineage differentiation using commercial differentiation formulations. Representative images of stained adipocytes [Oil Red O], osteocytes [Alizarin Red] and chondrocytes [Alcian Blue]. **hMSC cultured in MSC NutriStem® XF maintain their multilineage differentiation potential.**

Figure 11: Immunophenotyping of hMSC-AT

	Seeding concentration [cells/cm ²]						
	6000		5000		1000		
	FBS containing medium	MSC NutriStem® XF	FBS containing medium	MSC NutriStem® XF	FBS containing medium	MSC NutriStem® XF	
CD 34	0.31	0.91	0.16	0.3	0.52	1.32	0.17
CD 45	0.35	0.5	0.1	0.07	0.4	0.18	0.08
HLA-DR	1.54	0.3	0.91	0.11	7.16	0.16	2.27
CD 34+45+HLA-DR	0.73	0.57	0.39	0.16	2.69	0.55	0.84
CD 73	99.96	99.97	99.96	99.98	99.52	99.76	99.94
CD 90	99.98	99.99	99.97	99.9	99.94	99.6	99.95
CD 105	99.99	100	99.98	100	100	99.6	99.96
CD 44	99.93	100	99.98	99.94	99.8	99.8	99.62

Comparison FACS analysis results of hMSC-AT cultured for 3 passages in different seeding concentrations using SF, XF culture medium (MSC NutriStem® XF) vs. FBS-containing medium. **hMSC cultured in MSC NutriStem® XF maintain a classical profile of MSC markers with a lower percentage of hematopoietic contamination in all tested seeding concentrations.**

Figure 12: Genomic stability of hMSC



G-banding karyotyping analysis of hMSC-BM and AT expanded for 4 passages in MSC NutriStem® XF. **hMSC cultured in MSC NutriStem® XF maintain genomic stability.**

Summary

- hMSC from various sources can be efficiently isolated using MSC NutriStem® XF supplemented with 2% human AB serum (AT, BM) or w/o (PL).
- A higher number of hMSC was obtained after isolation using MSC NutriStem® XF in comparison to FBS-containing medium.
- Using MSC NutriStem® XF for isolation of hMSC enhances purity of MSC population in earlier passages [decreasing hematopoietic contamination] compared to initial isolation using FBS-containing medium.
- The highest proliferation rate of hMSC from a variety of sources was achieved using MSC NutriStem® XF in comparison to other commercially available SF media.
- MSC NutriStem® XF supports long-term culture of hMSC from a variety of sources.
- hMSC cultured in MSC NutriStem® XF retain the essential MSC characteristics (fibroblast-like morphology, surface markers phenotype, multilineage differentiation, self-renewal potential, and genomic stability).

The developed XF culture system (MSC NutriStem® XF medium, MSC Attachment Solution, MSC Dissociation Solution, Recombinant Trypsin Solution, MSC Freezing Solution) supports the initial isolation and long-term expansion of hMSC from various sources, suitable for cell therapy and tissue engineering.

Acknowledgments:

- * We would like to thank Professor Mark L. Weiss, Kansas State University, Department of Anatomy and Physiology, Manhattan, KS, for his invaluable contribution to this study.
- * We would like to thank Kasiak Research Pvt. Ltd., Maharashtra, India, for their cooperation in this study.

References:

1. Poster: ISCT 2012 Seattle, USA. Identification of optimal conditions for generating MSCs for preclinical testing: Comparison of three commercial serum-free media and low-serum growth medium. Yelica López, Elizabeth Trevino, Mark L. Weiss, Kansas State University, Department of Anatomy and Physiology, Manhattan, KS.
2. Poster: From the 23rd European Society for Animal Cell Technology (ESACT) Meeting: Better Cells for Better Health, Lille, France. 23-26 June 2013. Toward a serum-free, xeno-free culture system for optimal growth and expansion of hMSC suited to therapeutic applications. Mira Genser-Nir, Sharon Daniliuc, Marina Tevovsky, David Fiorentini, Biological Industries, Israel.
3. Seshareddy, K., Troyer, D., Weiss, M.L. Method to isolate mesenchymal-like cells from Wharton's jelly of umbilical cord. Methods Cell Biol. 2008; 86: 101-119.