A Novel Set of Serum-Free, Xeno-Free Differentiation Media for Adipogenesis, Osteogenesis and Chondrogenesis of Human Mesenchymal Stem Cells from Various Tissue Sources

Mira Genser-Nir, Sharon Daniliuc, Marina Tevrovsky, Roni Hazan Brill, Yuliya-Yael Miropolski, David Fiorentini.
Biological Industries, Beit Haemek, Israel.

Abstract

Human mesenchymal stem cells (hMSCs) are multipotent adult stem cells that can be isolated from various tissues as well as generated in vitro from hESCs and iPS cells.

hMSCs have at least trilineage differentiation potential in vitro (into fat, bone, and cartilage cells), and it is part of the Minimal Experimental Criteria for MSC proposed by the International Society for Cellular Therapy (ISCT). In addition, differentiation of hMSCs into specific lineage provides the basis for the use of human MSC in cell therapy applications. Either undifferentiated or differentiated, hMSCs can be used for in vivo implantation into damaged tissue sites. The differentiation potential may differ in relation to the culture condition and the source of hMSCs, and it is still unknown which source should be used for each specific disease.

The quality of the culture medium and differentiation media is particularly crucial with regard to therapeutic applications since multipotent hMSCs and differentiated cell properties can be significantly affected by medium components, culture condition, and the manual used for culturing and differentiation.

To date, most of the common differentiation media are composed of FBS and xenogenic compounds, which is a major drawback for both research and clinical applications. Improving the culture conditions and differentiation potential is required for differentiated hMSCs to become the cells of choice for use in modern regenerative medicine and drug screening.

The present study addressed the development of novel serum-free, xeno-free media and supplements that efficiently differentiate hMSCs from various sources into adipocytes, osteoblasts, and chondrocytes. Molecular characterization and functional assays were used to evaluate the quality and purity of the differentiated cells.

This novel SF, XF system enables achieving defined conditions for rapid generation of differentiated hMSCs towards tissue engineering and drug screening applications

Abbreviations

ACAN	Aggrecan
ARS	Alizarin Red S
CPC	Cetylpyridinium Chloride
FABP4	Fatty Acid Binding Protein 4
FBS	Fetal Bovine Serum
GAGs	Glycosaminoglycans
GuHCl	Guanidine HCL
hMSCs	hMSC Human Mesenchymal Stem Cells
hMSC-AT	Adipose Tissue-derived hMSC
hMSC-BM	Bone Marrow-derived hMSC
hMSC-DP	Dental Pulp-derived hMSC
hMSC-PL	Placenta-derived hMSC
hMSC-WJ	Wharton's Jelly (cord tissue)-derived hMSC
IF	Immunofluorescence
OCN	Osteocalcin (BGLAP)
PPARG	Peroxisome Proliferator - Activated Receptor
SF	Serum Free
XF	Xeno Free

Materials and Methods

Cells

hMSCs from a variety of sources: BM (Lonza, Promocell), AT (ATCC, Promocell), WJ (ATCC, Promocell) and DP (Lonza) were used in this study.

Culture system

hMSCs were cultured in a SF, XF expansion medium (MSC NutriStem® XF, BI) on pre-coated dishes (MSC Attachment Solution, BI). For expansion, cells were seeded at a concentration of 5000 viable cells/cm^2 and harvested using Recombinant Trypsin Solution (BI).

Differentiation assays

Adipogenesis and Osteogenesis: 6x10^4 cells/well were seeded in 24w/p (3-4x10^{4}cells/cm^2) in 0.5ml/wells of MSC NutriStem® XF, on pre-coated plates (MSC Attachment Solution, BI). The cells were incubated for 24 hrs. (37°C, 5% CO_2) to achieve >80% confluence following replacement of the culture medium with **MSCgo™** differentiation media (BI) or other desired differentiation media. Medium was changed according to the manufacturer instructions. After 10-24 days assay the cells were fixed for differentiation evaluation.

Chondrogenesis: for micromass culture, 1x10^5 cells/well were seeded in 96w/p U-bottom ULA plates (3-4x10^{5}cells/cm^2) in 0.1ml of MSC NutriStem® XF (BI). The cells were incubated for 24 hrs. (37°C, 5% CO_2) to achieve spheroids following replacement of the culture medium with **MSCgo™ Chondrogenic** (BI) or other desired chondrogenic differentiation media. Medium was changed according the manufacturer instructions. After 14-28 days assay the cells were fixed for differentiation evaluation.

Evaluation of differentiation

Morphology evaluation was performed for observation of mature adipocytes cells composed of oil droplets (adipogenesis), to detect the formation of mineralized culture with calcified nodules and calcium secretion (osteogenesis) and to evaluate the shape and size of the cartilage-like spheroid structure (chondrogenesis).

Histochemical staining: following differentiation, cells were fixed (4% formaldehyde) and incubated with typical staining solutions:
Adipogenesis: 0.2% Oil Red O (Sigma) for 30 min. Positive red staining of oil droplet accumulation in the cells indicates mature adipocytes.
Osteogenesis: 2% ARS (Sigma) for 30 min. Positive red staining of calcium indicates mature osteocytes.
Chondrogenesis: 1% Alcian Blue (Sigma) for 24 hrs. Positive blue staining of proteoglycans indicates mature chondrocytes.

Semi-quantification of staining intensity:
Adipogenesis: Oil Red O staining was quantified by eluting the dye in 2-propanol (100%) for 1 hr. and measuring absorbance at 550 nm.
Osteogenesis: Alizarin Red staining was quantified by eluting the dye in 10% (w/v) cetylpyridinium chloride (CPC) for 1 hr., and measuring absorbance at 550 nm.
Chondrogenesis: Alcian Blue staining was quantified by eluting the dye in 6M Guanidine HCL (GuHCl) for 24 hrs. (2-8°C) and measuring absorbance at 600 nm.

Histopathological Examinations: For assessment of Chondrogenic differentiation, tissue sections at approximately 5 microns thickness were formalin-fixed (4% formaldehyde), paraffin-embedded, and stained with Toluidine blue using routine protocol.

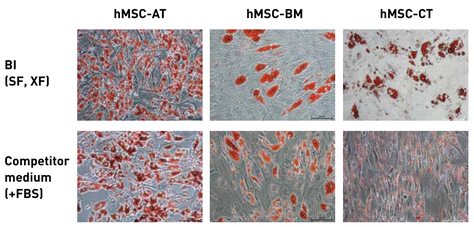
RT-qPCR analyses: total RNA was extracted (ReliaPrep™ RNA Cell Miniprep System, Promega), and reverse transcribed (EZ-First Strand cDNA, BI). Quantitative real-time PCR was performed using TaqMan® Universal PCR Master Mix (Applied Biosystems), gene-specific TaqMan PCR probes and FAM primers for matured genes, specific for each cell lineage.
Adipogenesis: FABP4 and PPARG.
Osteogenesis: RUNX2, SP7, ALP, Osteonectin (SPOCK2), and Osteocalcin (OCN).
Chondrogenesis: ACAN and COL10A1.
Each sample was tested in triplicates. The mRNA expression levels were normalized to the levels of undifferentiated hMSCs for matured genes, specific for each cell lineage.

Immunofluorescence (IF) staining: following differentiation, cells were fixed (4% formaldehyde) and immune stained as follow. The nuclei were counterstained with Dapi (Blue, MP Bioscience).
Adipogenesis: Fatty acid binding protein 4 (FABP4) was detected using Goat Anti-Mouse/Rat FABP4 polyclonal antibody (AF1443, R&D Systems) at 10 µg/mL for 24 hrs. at 2-8°C, following staining with Rhodamine Red™-X conjugated donkey anti-goat IgG secondary antibody (red, Jackson).
Osteogenesis: OCN (BGALP) was detected using Mouse Anti Human/Rat OCN monoclonal antibody (MAB1419, R&D Systems) at 10 µg/mL for 24 hrs. at 2-8°C following staining with Alexa Fluor® 488-conugated donkey anti-mouse IgG secondary antibody (green, Jackson).
Chondrogenesis: ACAN (Aggrecan) was detected using Goat Anti-Human Aggrecan Polyclonal Antibody (AF1220, R&D Systems) at 10 µg/mL for 24 hrs. at 2-8°C, following staining with Rhodamine Red™-X donkey anti-goat IgG secondary antibody (red, Jackson).

Results

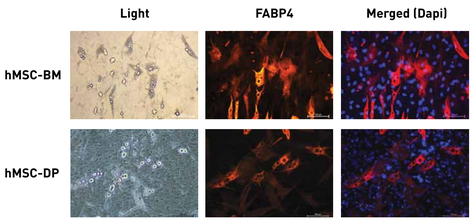
I. Adipogenesis

Fig 1: **MSCgo™ Adipogenic is superior for a variety of sources of hMSCs**



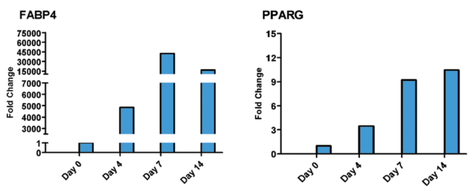
hMSCs derived from a variety of sources: AT, BM , and CT, seeded in SF, XF culture system (MSC NutriStem® XF and MSC Attachment Solution) for 24 hrs. following initiation of adipogenesis using MSCgo™ Adipogenic and commercial medium composed of FBS. Representative images (x200) of stained adipocytes (Oil Red O) after 11 days (hMSC-AT), 14 days (hMSC-BM) and 17 days (hMSC-CT) of adipogenesis assay.
Adipogenesis of hMSCs from various sources is achievable under a SF, XF culture system.

Fig 2: **High expression level of mature adipogenic marker (IF)**



Light microscopic and immunofluorescence staining (FabP4,red) counterstained with DAPI (blue), after 15 days (hMSC-BM) and 24 days (hMSC-DP) of adipogenesis assay in MSCgo™ Adipogenic.
hMSCs differentiated into adipocytes using MSCgo™ Adipogenic Medium exhibit high expression of mature adipogenic markers.

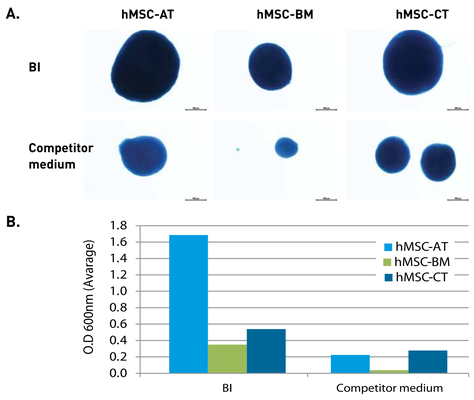
Fig 3: **Elevated expression of adipocyte markers during adipogenesis**



Real-time results of adipogenic differentiated hMSC-AT during adipogenesis assay using MSCgo™ Adipogenic. The results are expressed as a fold-change of expression level according to non-differentiated hMSC-AT (Day 0).
High expression of the adipocyte-related genes is observed during adipogenesis of hMSCs using MSCgo™ Adipoogenic.

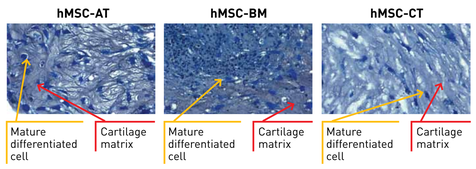
II. Chondrogenesis

Fig 4: **MSCgo™ Chondrogenic is superior for a variety of sources of hMSCs**



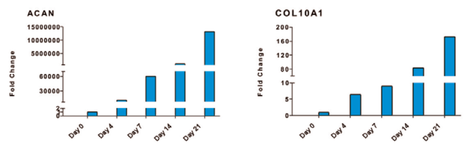
hMSCs derived from a variety of sources induced to chondrogenesis using MSCgo™ Chondrogenic and commercial medium composed of FBS.
A. Representative images (x40) of stained chondrocytes (Alcian Blue) after 21 days of chondrogenesis assay. **B.** Semi quantitative results for the Alcian Blue intensity staining. **Chondrogenesis of hMSCs from various sources is superior using MSCgo™ Chondrogenic, results in larger cartilage with a higher intensity of stained GAGs in comparison to competitor medium.**

Fig 5: **Evaluation of cartilage maturation by histology examination**



Histopathological results of hMSCs from various sources stained with Toluidine Blue after 21-day chondrogenesis assay using MSCgo™ Chondrogenic. The presence of a cartilage matrix and mature hypertrophic chondrocytes (lacunae) are indicated by positive staining of proteoglycans with Toluidine Blue. **MSCgo™ Chondrogenic efficiently induces hMSCs from various sources to differentiate into mature chondrocytes surrounded by cartilage matrix.**

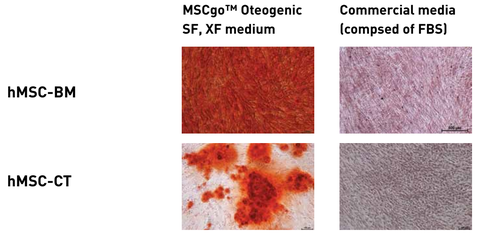
Fig 6: **Elevated expression of chondrocyte markers during chondrogenesis of hMSCs**



Real-time results of ACAN and collagen type X alpha 1 (COL 10A1) in chondrogenic differentiated hMSC-AT during chondrogenesis assay using MSCgo™ Chondrogenic. The results are expressed as a fold-change of expression level according to non-differentiated hMSC-AT (Day 0). **Elevated expression of the mature chondrocyte-related genes is observed during chondrogenesis of hMSCs using MSCgo™ Adipoogenic.**

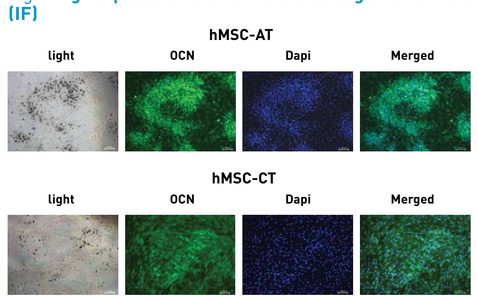
III. Osteogenesis

Fig 7: **MSCgo™ Osteogenic is superior for a variety of sources of hMSCs**



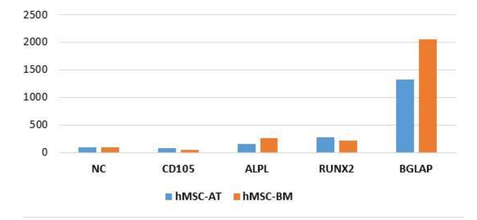
Representative images (x100) of ARS stained osteocytes after 11 days (hMSC-BM) and 14 days (hMSC-WJ) of osteogenesis assay in MSCgo™ Osteogenic and commercial media composed of FBS.
MSCgo™ Osteogenic is suitable for a variety of hMSC types, whereas different commercial media (composed of FBS) are not.

Fig 8: **High expression level of mature osteogenic markers (IF)**



Light microscopic and immunofluorescence staining (OCN, green) of hMSCs, counterstained with DAPI (blue), after 24 days of osteogenesis in MSCgo™ Osteogenic. **hMSCs differentiated into osteocytes using MSCgo™ Osteogenic exhibit calcified culture with high expression of mature osteogenic markers.**

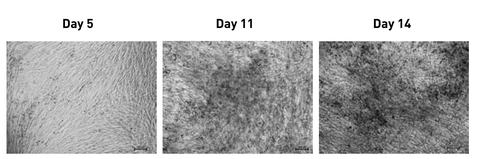
Fig 9: **Elevated expression of mature osteogenic markers (RT-PCR)**



Relative expression (RT-PCR) of osteocytes markers after 10 days of osteogenesis in MSCgo™ Osteogenic in comparison to un-differentiated hMSCs (Cultured in MSC NutriStem® XF). **Osteogenic markers were up-regulated whereas un-differentiated hMSC marker (CD-105) was down regulated using MSCgo™ Osteogenic.**

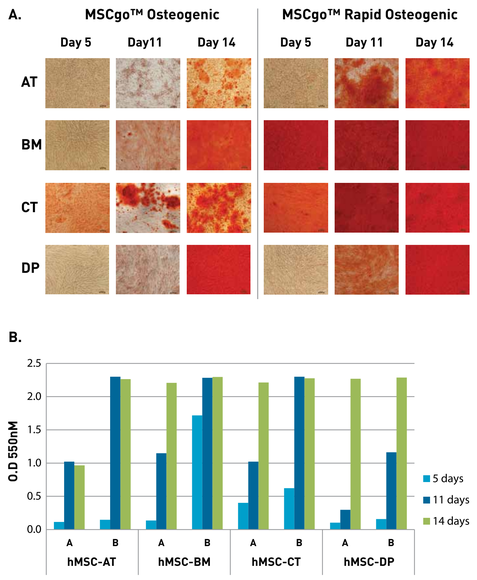
IV. Rapid Osteogenesis

Fig 10: **Mineralization kinetics of hMSC-CT differentiated in MSCgo™ Rapid Osteogenic**



hMSC-CT seeded in SF, XF culture system (MSC NutriStem® XF and MSC Attachment Solution) for 24 hrs. following initiation of osteogenesis using MSCgo™ Rapid Osteogenic.
Representative images (x100) of osteogenic differentiated hMSC-CT after 5, 11 and 14 days in MSCgo™ Rapid Osteogenic.
Rapid mineralization is observed using MSCgo™ Rapid Osteogenic.

Fig 11: **Rapid osteogenesis is achievable using MSCgo™ Rapid Osteogenic**

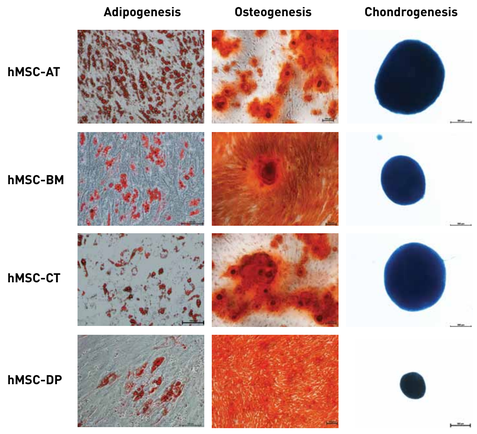


A. MSCgo™ Osteogenic
B. MSCgo™ Rapid Ostogenic

Kinetics of mineralization during osteogenesis of hMSCs from various sources using MSCgo™ Osteogenic and MSCgo™ Rapid Osteogenic.
A. ARS stained osteocytes (x100) after 5, 11, and 14 days of osteogenesis assay. **B.** Semi quantitative results for the ARS intensity staining.
At the same time points, advanced osteocytes maturation is observed using MSCgo™ Rapid Osteogenic in comparison to MSCgo™ Osteogenic, results in higher level of mineralization with positive correlation to the intensity staining of ARS.

Summary

Fig 12: **Suitability for various sources of hMSCs**



hMSCs from various sources differentiated into adipocytes (X200, Oil Red O), osteocytes (X100, ARS) and chondrocytes (x40, Alcian Blue) using BI MSCgo™ differentiation media (SF,XF). Adipogenesis: 16-24-day assay, osteogenesis: 11-15-day assay, chondrogenesis: 18-24-day assay.
Efficient differentiation of hMSCs from various sources into adipocytes, osteocytes, and chondrocytes is achievable using a novel set of SF, XF differentiated media (BI).

- hMSCs from various sources can be efficiently differentiated into adipocytes, osteocytes, and chondrocytes under SF, XF culture conditions using the MSCgo™ differentiation media, respectively.
- Superior maturation of differentiated hMSCs can be achieved under SF, XF using the MSCgo™ differentiation media in comparison to competitor media.
- Rapid osteogenic maturation with intensive mineralization can be achieved using the MSCgo™ Rapid Osteogenic.

To conclude: The present study addressed the development of novel serum-free, xeno-free differentiation media that efficiently differentiate hMSCs from various sources into adipocytes, osteocytes, and chondrocytes.
This novel SF, XF system enables achieving defined conditions, for rapid generation of differentiated hMSCs towards tissue engineering and drug screening applications.

E7/1 4/16