

Assuring Multipotency of human Mesenchymal Stem Cells (hMSC)

Introduction

Over the past decade, stem cell research has provided new avenues for deeper investigation into tissue repair and aging processes, as well as regenerative medicine methods. One of the major players in such research endeavors are mesenchymal stem cells (MSC), also known as mesenchymal stromal cells. MSC are typically found in bone marrow, adipose, placental, and umbilical cord tissues¹ and are a type of adult stem cell. In vivo, these cells are headquartered in special microenvironments or “niches” in the body where they remain quiescent until triggered to self-renew to maintain their stem cell population or to differentiate into specialized daughter cell types. MSC may also remain quiescent for extended periods of time. Unlike pluripotent stem

cells (e.g., embryonic stem cells) that can specialize into numerous cell types, MSC are non-hematopoietic, multipotent stem cells and thus possess a limited differentiation capacity that yields fewer specified cell types. MSCs produce bone, cartilage, fat, and stromal cell types when required to facilitate tissue growth or repair². As such, regenerative medicine and biomedical research often employ human MSCs (hMSC) in studies on connective tissue repair³, immune response-based diseases⁴, and inflammatory diseases⁵ to better understand etiology and assess treatment approaches. The use of hMSCs in research, however, is not without its particular challenges. Like all adult stem cells, hMSC populations are found in small quantities in situ

and can be technically challenging to isolate from tissue. Moreover, freshly harvested hMSC populations may also contain other, off-target cell types. Thus, hMSCs must be purified and expanded in vitro to reach sufficient quantities for experimentation. This must be judiciously done as differentiation potential and genomic integrity begin to wane around 5-12 passages^{6,7}. Researchers may use the phenotypic and functional assessments described here to assist in successful purification and correct identification of hMSC identity. When combined with a high-performance culture medium, such methods encourage hMSC culture integrity necessary to generate good-quality, reproducible research, and cell-based therapy.

Methods

Phenotypic Analysis: Morphology

Adherence to plastic culture dish is a well-documented characteristic of MSCs cultured in serum-containing media. Harvested cells are established in culture by flushing out cells directly onto plastic dishes⁸. First employed by Friedenstein and colleagues in 1970⁹, this harvest method is recommended as an initial parameter for MSC identification¹⁰. Once in culture, hMSC display a spindly, fibroblast-like morphology (Fig. 1).

Cellular morphology, however, does not guarantee a pure cell population identity. Some hematopoietic cells can adhere to plastic, though these are typically lost over multiple passages and media changes. To further confirm hMSC identity, additional methods of characterization using proteomic and functional approaches are required.

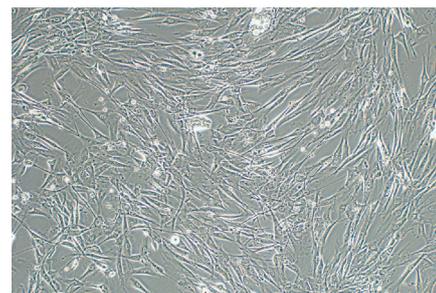


Figure 1: hMSCs demonstrate a spindle, fibroblast-like morphology in culture.

Phenotypic Analysis: Cell Surface Markers

Cell sorting-equipped flow cytometers readily capitalize upon the presence of hMSC cell surface markers to purify cultures and provide confirmation of culture identity. For the most accurate results, both positive and negative cell surface markers should be used (Fig. 2). A first pass sorting attempt that only uses negative surface markers may be helpful to maximize non-MSCs exclusion from the harvested hMSC population. At least 95% of the purified hMSC culture must express CD105, CD73, and CD90 and less than 2% of the cell population should express CD45, CD34, CD14, CD11b, CD79 α , CD19, or HLA class II¹⁰. Multicolor analyses involving two or more surface marker stains should also be used to validate cell identity whenever possible. Though cell surface markers may be qualitatively evaluated via immunohistochemical or immunofluorescent approaches, a quantitative method like flow cytometry

is best for a snapshot of overall culture quality. Following up with karyotypic analysis is recommended for freshly

purified MSC cultures to prevent experimental complications due to genomic irregularity.

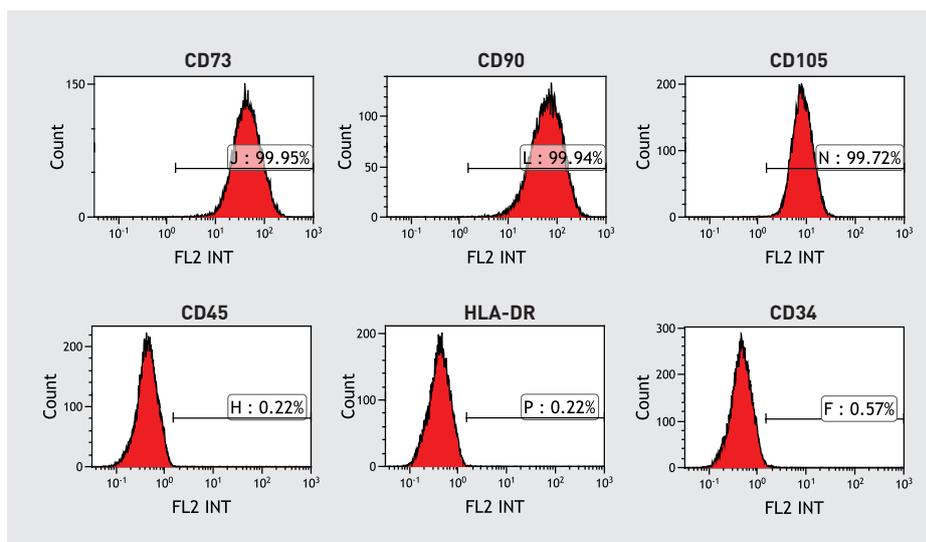


Figure 2: Confirmation of MSC identity via cell surface marker profiles in adipose-derived hMSCs. Isolated cells were seeded on plates coated with Biological Industries MSC Attachment Solution and cultured in MSC NutriStem[®] XF medium supplemented with 2% human AB serum.

Functional Analysis: Differentiation Capacity

MSCs are identified by their multipotent capacity to differentiate into osteoblasts, chondrocytes, and adipocytes when exposed to the correct signal combinations. As follows, *in vitro* differentiation and subsequent tissue validation is necessary to validate both MSC identity and multipotent integrity. hMSC differentiation protocols to produce each cell type are readily found in published literature for this purpose. For easy and reproducible results,

cell type-specific hMSC differentiation media like the MSCgo[™] series (Fig. 3) may also be used in functional assessments. Histological or immuno-based stains of differentiated cells are recommended to confirm the cells identity. Qualitative confirmation of MSC differentiation capacity is required at minimum, though flow cytometry may be used to quantitate MSC differentiation efficacy.



Figure 3: Commercial differentiation media such as MSCgo[™] differentiation media may be used to encourage MSCs to differentiate into osteocytes, chondrocytes, or adipocytes.

Osteoblasts

Osteoblasts present a generally round shape and form bone by secreting an osteoid bone matrix that eventually becomes calcified. Osteoblast histochemical stains like Alizarin Red S (Fig. 4) and Von Kossa bind to calcium in these matrices and are therefore

useful for confirming osteoblast identity. Immunohistochemical assessment with osteoblast-specific markers such as osteocalcin (mature osteoblasts) and RUNX2/CBFA1 (differentiating osteoblasts) may be also be used to confirm osteoblast identity.

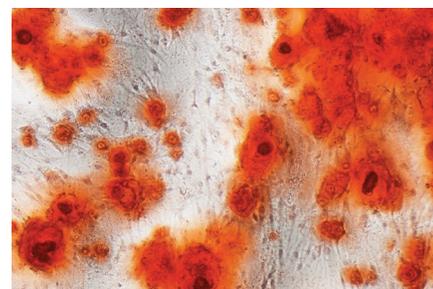


Figure 4: Alizarin Red verifies osteoblast identity by binding directly to the calcium found in osteoblasts' secreted bone matrix.

Chondrocytes

Chondrocytes are physically characterized by their round shape and undefined cytoplasmic borders. Like osteoblasts, chondrocytes secrete extracellular matrix molecules—typically, collagens and proteoglycans. The Alcian Blue (Fig. 5) histochemical

stain binds to the acidic polysaccharides (e.g., glycosaminoglycans) produced by chondrocytes. Immuno-driven staining methods may be used to further confirm chondrocyte identity. Some recommended chondrocyte-specific markers include collagen II and aggrecan.

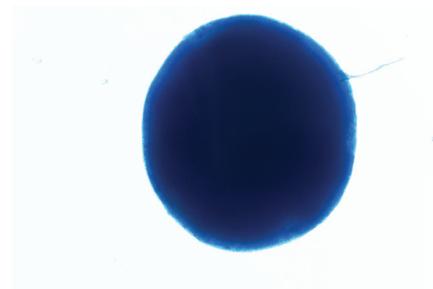


Figure 5: Alcian Blue binds to acidic polysaccharides associated with chondrocyte function.

Adipocytes

Adipocytes have a very distinct morphology that features a large, centrally-located lipid vacuole, narrow cytoplasm, and small, peripherally located nuclei. The fat-soluble dye, Oil Red O (ORO), may be used to confirm successful adipocyte differentiation.

When exposed to adipocytes, ORO stains the lipid vacuoles bright red (Fig. 6). Instead of or in addition to ORO, an immune-based stain with adipocyte-specific markers—such as PREF-1 (pre-adipocytes), FABP4 (mature adipocytes), and adiponectin (adipocytokine specific to adipocytes)—may be used.

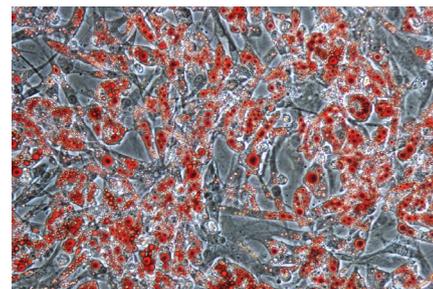


Figure 6: Adipocytes are readily detected by Oil Red O, a histochemical stain that binds to the large lipid vacuoles found in fat cells.

Functional Analysis: Colony Forming Unit-Fibroblast Assay

In addition to differentiation capacity, hMSC self-renewal aptitude should also be assessed. The Colony Forming Unit-Fibroblast (CFU-F) Assay measures hMSC self-renewal potential by investigating the efficiency by which low density cells

produce spindle-shaped colonies. Plated cells are allowed to grow for 2-3 weeks before distinct colonies are counted to calculate CFU-F efficiency (Fig. 7). High quality hMSCs are expected to return a high CFU-F efficiency.

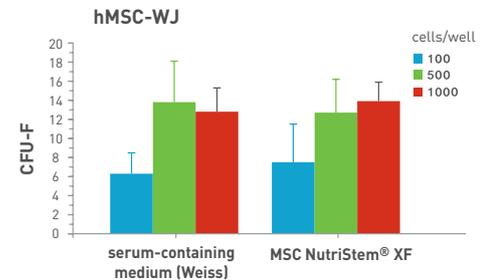


Figure 7: The CFU-F Assay calculates hMSC cultures' self-renewal capacity. Here, hMSCs were seeded at three different concentrations at the beginning of the assay.

Summary

Following characterization of hMSC identity and quality, cultures may be scaled up for intended applications. hMSCs should always be evaluated (Fig. 8) following isolation, thawing, and at regular intervals between passages to confirm

the best starting cell quality. High hMSC quality is critical for study reproducibility as well as on-target differentiation when specialized cell types are desired. To this end, using the methods described above and a reliable maintenance medium,

such as MSC NutriStem® XF, will ensure propagation of high-quality hMSC cultures that maintain their capacity to self-renew and differentiate into multiple lineages.

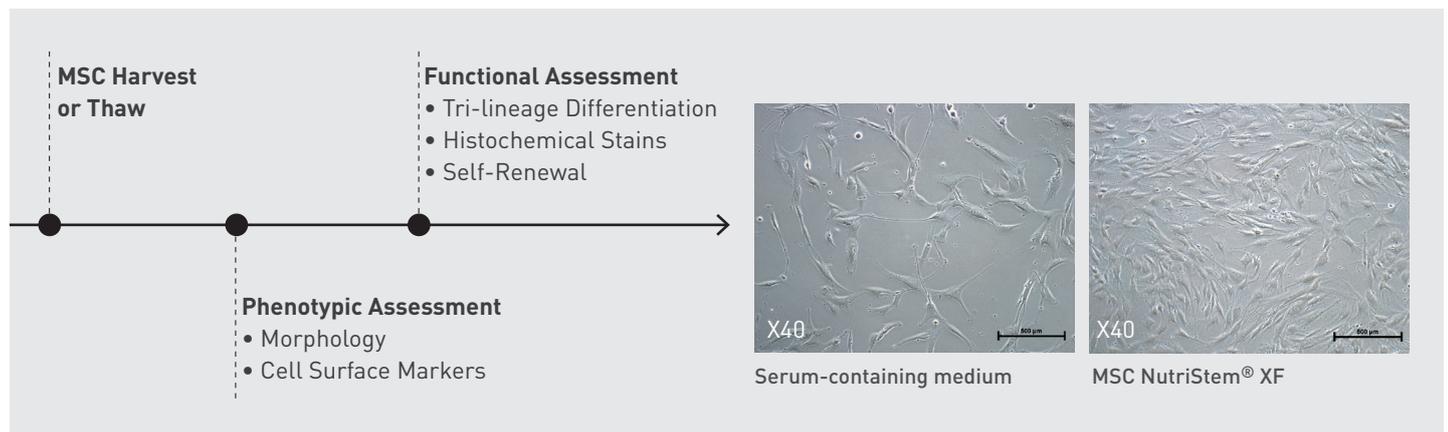


Figure 8: Flowchart of MSC validation process. Reliable hMSC isolation and expansion medium is critical for successful isolation and expansion of high-quality hMSCs. For best results, use a reliable culture medium that has been optimized for hMSC culture, such as MSC NutriStem® XF.

Product	Cat.#	Size
MSC NutriStem® XF Basal Medium	05-200-1A	500ml
MSC NutriStem® XF Supplement Mix	05-201-1U	3ml
MSCgo™ Osteogenic Differentiation Medium	05-440-1B	100ml
MSCgo™ Rapid Osteogenic Differentiation Medium	05-442-1B	100ml
MSCgo™ Chondrogenic Differentiation Basal Medium	05-220-1B	100ml
MSCgo™ Chondrogenic Differentiation Supplement Mix	05-221-1D	10ml
MSCgo™ Adipogenic Differentiation Basal Medium	05-330-1B	100ml
MSCgo™ Adipogenic SF, XF Supplement Mix I	05-331-1-01	0.1ml
MSCgo™ Adipogenic SF, XF Supplement Mix II	05-332-1-15	1.5ml

Citations

- Hendijani, F. Explant culture: An advantageous method for isolation of mesenchymal stem cells from human tissues. *Cell Prolif* **50**, n/a-n/a (2017)
- Pittenger, M. F. *et al.* Multilineage Potential of Adult Human Mesenchymal Stem Cells. *Science* **284**, 143–147 (1999).
- Kharaziha, P. *et al.* Improvement of liver function in liver cirrhosis patients after autologous mesenchymal stem cell injection: a phase I–II clinical trial. *European Journal of Gastroenterology & Hepatology* **21**, 1199 (2009).
- Kebriaei, P. *et al.* Adult human mesenchymal stem cells added to corticosteroid therapy for the treatment of acute graft-versus-host disease. *Biol. Blood Marrow Transplant.* **15**, 804–811 (2009).
- Koh, Y.-G. *et al.* Mesenchymal stem cell injections improve symptoms of knee osteoarthritis. *Arthroscopy* **29**, 748–755 (2013).
- Wagner, W. *et al.* Replicative Senescence of Mesenchymal Stem Cells: A Continuous and Organized Process. *PLOS ONE* **3**, e2213 (2008).
- Halfon, S., Abramov, N., Grinblat, B. & Ginis, I. Markers distinguishing mesenchymal stem cells from fibroblasts are downregulated with passaging. *Stem Cells Dev.* **20**, 53–66 (2011).
- Schipani, E. & Kronenberg, H. M. Adult mesenchymal stem cells. in [Harvard Stem Cell Institute, 2008].
- Friedenstein, A. J., Chailakhjan, R. K. & Lalykina, K. S. The development of fibroblast colonies in monolayer cultures of guinea-pig bone marrow and spleen cells. *Cell Tissue Kinet* **3**, 393–403 (1970).
- Dominici, M. *et al.* Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. *Cytotherapy* **8**, 315–317 (2006).
- Hofer, E. L. *et al.* Mesenchymal Stromal Cells, Colony-Forming Unit Fibroblasts, From Bone Marrow of Untreated Advanced Breast and Lung Cancer Patients Suppress Fibroblast Colony Formation From Healthy Marrow. *Stem Cells and Development* **19**, 359–370 (2009).