Osteogenic differentiation and analysis of MSC

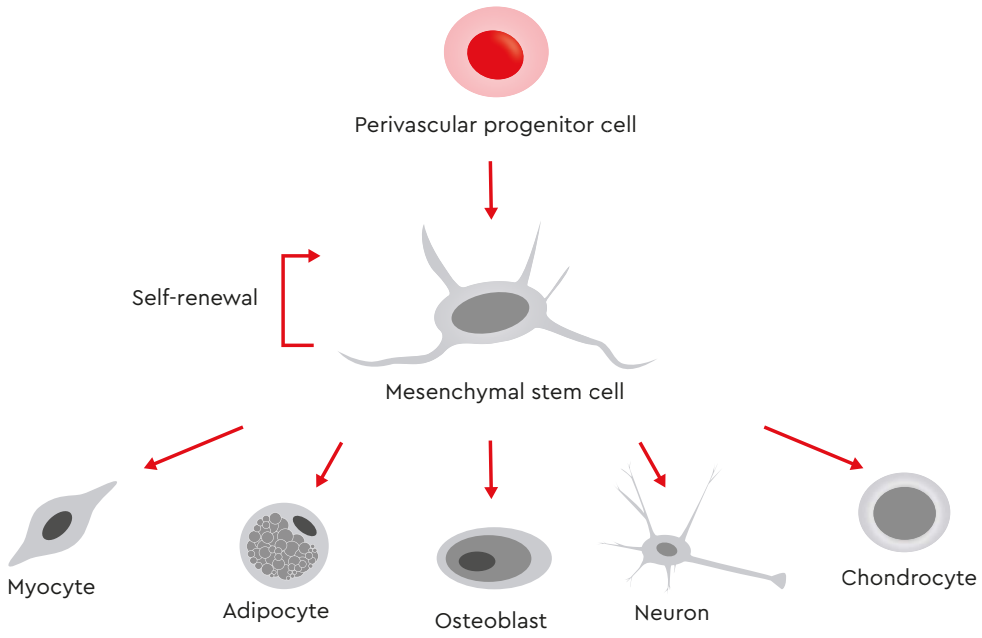
Application note

Background

Mesenchymal stem cells (MSC) are fibroblastoid multipotent adult stem cells with a high capacity for self-renewal. So far, these cells have been isolated from several human tissues, including bone marrow, adipose tissue, umbilical cord matrix, tendon, lung, and the periosteum [1]. Recently it has been shown that MSC originate from the perivascular niche, a tight network present throughout the vasculature of the whole body. These perivascular cells lack endothelial and hematopoietic markers, e.g., CD31, CD34 and CD45, but express CD146, PDGF-R beta, and alkaline phosphatase [2].

According to the position paper published by the International Society for Cellular Therapy (ISCT), MSC express the surface markers CD73, CD90 and CD105 and stain negative for CD14 or CD11b, CD34, CD45, CD79α or CD19, and HLA-DR [3]. In addition to surface marker analysis, the most common and reliable way to identify a population of MSC is to verify their multipotency. MSC can differentiate into adipocytes, osteoblasts, myocytes, and chondrocytes *in vivo* and *in vitro* [1,4]. Transdifferentiation of MSC into cells of non-mesenchymal origin, such as hepatocytes, neurons and pancreatic islet cells, has also been observed *in vitro* when specific culture conditions and stimuli are applied [1].

The directed differentiation of MSC is carried out *in vitro* using appropriate differentiation media, such as our ready-to-use MSC Differentiation Media (see below for differentiation protocol). Terminally differentiated cells are histochemically stained to determine their respective identities (see below for staining protocol).



Marker

Perivascular progenitor cell

- CD146⁺
- PDGF Receptor-β⁺
- Alkaline Phosphatase⁺
- CD31⁻
- CD34⁻

Mesenchymal stem cell

- CD73⁺
- CD90⁺
- CD105⁺
- CD14⁻ or CD11b⁻
- CD34⁻
- CD45⁻
- CD19⁻ or CD79α⁻
- HLA-DR⁻

Protocol for osteogenic differentiation and analysis of MSC

I. Differentiation protocol

Materials

- Human Mesenchymal Stem Cells from bone marrow (hMSC-BM, C-12974)
- Mesenchymal Stem Cell Growth Medium 2 (C-28009)
- Mesenchymal Stem Cell Osteogenic Differentiation Medium (C-28013)
- Collagen Type I precoated 6-well plate (e.g. Gibco™ Cat. No. 12037556)

Use aseptic techniques and a laminar flow bench.

1

Seed mesenchymal stem cells

Bring MSC Growth Medium 2 (C-28009) to room temperature and pipet 3 ml medium per well in a collagen type I precoated 6-well plate. Plate MSC with a seeding density of 5,000 cells/cm² in each well, e.g. 48,000 cells per well, using MSC Growth Medium 2. Let the cells grow. Work in duplicate.

Important: Allow the cells to reach at least 80–90% confluency. This will take 4–6 days.

2

Induce osteogenic differentiation

Induce one of the duplicate samples with 3 ml MSC Osteogenic Differentiation Medium (C-28013). Use 3 ml MSC Growth Medium 2 for the remaining well as a negative control. Incubate for 15 days. Change the medium every 2–3 days (e.g. Monday, Wednesday, Friday). Be careful not to disturb the cell monolayer.

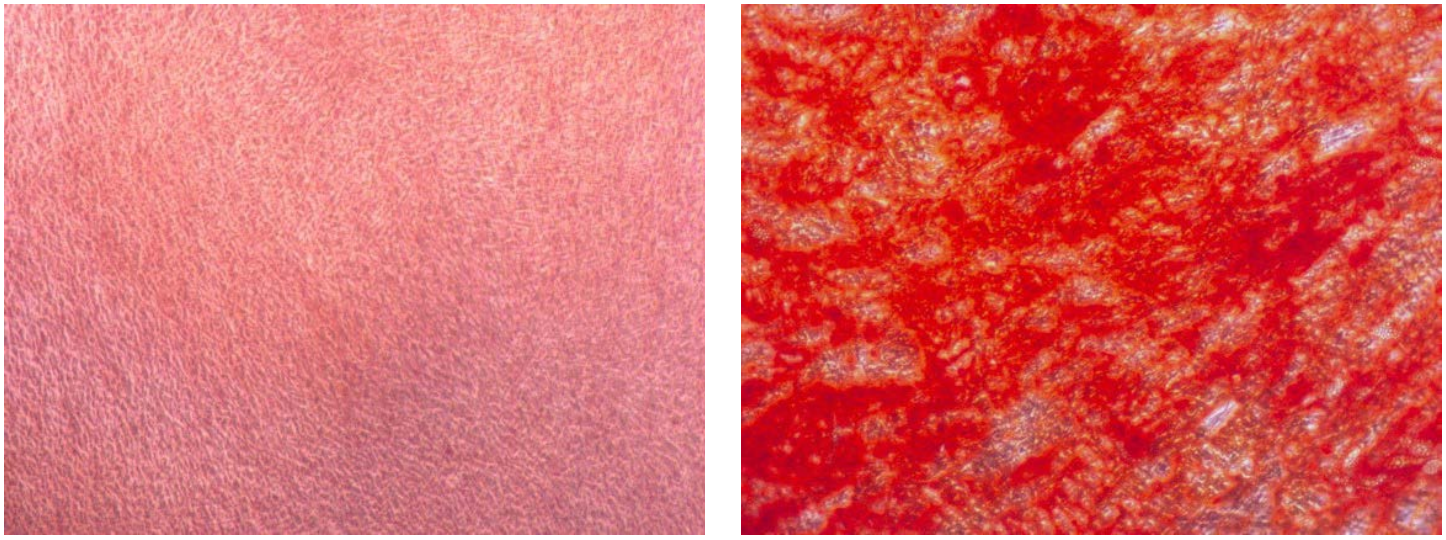


Fig. 1: Alizarin Red S staining of extracellular calcium deposits in mineralized hMSC-BM (human MSC derived from bone marrow)-derived mature osteoblasts. *Cells were cultured for 15 days in MSC Growth Medium 2 (C-28009) for the negative control (left image) or MSC Osteogenic Differentiation Medium (C-28013) for the differentiation sample (right image). The cells were fixed and afterwards stained with 2% Alizarin Red solution. In contrast with the negative control, the mature osteoblasts differentiated from MSC show intense red-orange staining of mineralized bone matrix. Note also the concentration of Alizarin Red S staining in some of the larger bone nodules.*

II. Osteoblast detection (Calcium deposits)

Materials

- Saccomanno Fixation Solution (Morphisto, Cat. No. 13881.00250)
- Alzarin Red S (Merck KGaA, Cat.No. A5533)
- Dulbecco's phosphate-buffered saline (PBS) without Ca^{++}/Mg^{++} (C-40232)
- Syringe filter, 0.22 µm

Important: Do not let the cells dry for longer than 30 seconds throughout the entire staining procedure

In contrast to undifferentiated MSC, differentiated osteoblasts accumulate vast extracellular calcium deposits (mineralization). This process is accompanied by the formation of bone nodules (Fig. 1).

Osteoblast-mediated mineralization is therefore indicative of the formation of bone mass and can be specifically detected using the bright orange-red dye, Alizarin Red S.

1

Prepare solutions and buffers

To prepare the Alizarin Red S 2% staining solution dissolve 2 g Alizarin Red S in 90 ml distilled water, mix and adjust the pH to 4.1–4.3 with hydrochloric acid, as necessary. Then, bring up to a final volume of 100 ml with distilled water and filter (0.22 µm) the dark-brown solution. Store at 2–8 °C in the dark for up to 2 months.

Note: The correct pH of the solution is critical. Check pH (at ambient temperature) if the solution is more than 1 month old.

2

Wash the cells

Remove the cells from the incubator and carefully aspirate the medium. Gently wash the cells with Dulbecco's phosphate-buffered saline (PBS) without Ca^{++}/Mg^{++} (C-40232).

Note: Do not disrupt the cell monolayer!

3

Fix the cells

Carefully aspirate the PBS and add enough Saccomanno Fixation Solution to cover the cellular monolayer. After at least 60 minutes at room temperature gently aspirate the fixation solution and wash the cells with 3 ml distilled water.

4

Stain the cells

Immediately before use, pass the required amount of Alizarin Red S staining solution through a 0.22 µm syringe filter equipped with a PES-membrane to get rid of precipitates. Carefully aspirate the distilled water and add enough filtered Alizarin Red S staining solution to cover the cellular monolayer. Incubate at room temperature in the dark for 10–15 minutes. Monitor staining progress for 10 minutes and stop the process when staining intensity is sufficient. Negative control should be colorless-yellowish and differentiated cells orange-red.

5

Wash the cells

Carefully aspirate the Alizarin Red S staining solution and wash the cell monolayer four times with 3 ml distilled water. Carefully aspirate the distilled water and add fresh distilled water to cover the cell layer.

6

Analyze the cells

Analyze the sample immediately, as the dye may bleed upon prolonged storage without embedding. Undifferentiated MSC (without extracellular calcium deposits) are colorless or slightly purple, whereas MSC-derived osteoblasts (with extracellular calcium deposits) stain bright orange-red (Fig. 1).

III. Osteoblast detection (Alkaline phosphatase)

Materials

- BCIP/NBT tablets (SigmaFast™ BCIP-NBT, Sigma Aldrich)
- Tween 20
- Saccomanno Fixation Solution (Morphisto, Cat. No. 13881.00250)
- Dulbecco's phosphate-buffered saline (PBS) without Ca^{++}/Mg^{++} (C-40232)
- Syringe filter, 0.22 µm

Important: Do not let the cells dry for longer than 30 seconds throughout the entire staining procedure

Undifferentiated MSC show weak alkaline phosphatase (AP) activity, whereas differentiated osteoblasts display very high AP activity. AP activity is therefore an indicator of successful differentiation of MSC into osteoblasts*. AP can be detected easily using BCIP/NBT (5-Bromo-4-chloro-3-indolyl phosphate/Nitro blue tetrazolium) as a substrate, which stains cells blue-violet when AP is present.

**AP activity is not limited to osteoblasts. Therefore, a second confirmation, e.g. direct staining of extracellular calcium deposits (mineralization), may be necessary to confirm differentiation of MSC into osteoblasts.*

1

Prepare solutions and buffers

Dissolve one BCIP/NBT tablet in 10 ml distilled water to prepare the substrate solution. Store in the dark and use within 2 hours. Add 0.05% Tween 20 to PBS without Ca^{++}/Mg^{++} to prepare the washing buffer.

2

Wash the cells

Remove the cells from the incubator and carefully aspirate the medium. Gently wash the cells with PBS.

Note: Do not disrupt the cell monolayer.

3

Fixation the cells

Carefully aspirate the PBS and add enough Saccomanno Fixation Solution to cover the cellular monolayer. After 60–90 seconds gently aspirate the fixation solution and wash the cells with the washing buffer.

Note: Longer fixation will lead to irreversible inactivation of AP.

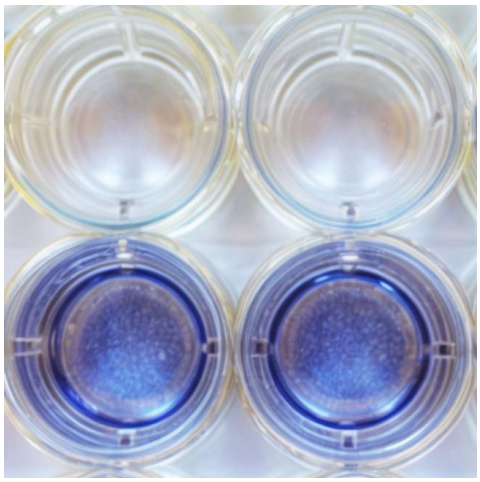


Fig. 2: Undifferentiated MSC *(AP negative, upper row) are colorless or faintly bluish, whereas MSC-derived osteoblasts (AP positive, lower row) are dark blue-violet. The higher the AP activity, the more intense the color.*

4

Stain the cells

Carefully aspirate the washing buffer and add enough BCIP/NBT substrate solution to cover the cellular monolayer. Incubate at room temperature in the dark for 5–10 minutes. Check staining progress every 2–3 minutes.

5

Wash the cells

Carefully aspirate the substrate solution and wash the cell monolayer with washing buffer. Then aspirate the washing buffer and add PBS.

6

Analyze the cells

Evaluate the staining results. Refer to Fig. 2 for an example of AP detection.

Related products

Product	Size	Catalog number
Human Mesenchymal Stem Cells from Bone Marrow (hMSC-BM)	500,000 cryopreserved cells 500,000 proliferating cells	C-12974 C-12975
Human Mesenchymal Stem Cells from Umbilical Cord Matrix (hMSC-UC)	500,000 cryopreserved cells 500,000 proliferating cells	C-12971 C-12972
Human Mesenchymal Stem Cells from Adipose Tissue (hMSC-AT)	500,000 cryopreserved cells 500,000 proliferating cells	C-12977 C-12978
Mesenchymal Stem Cell Growth Medium 2 (Ready-to-use)	500 ml	C-28009
Mesenchymal Stem Cell Growth Medium XF (Ready-to-use)	500 ml	C-28019
Mesenchymal Stem Cell Adipogenic Differentiation Medium 2 (Ready-to-use)	100 ml	C-28016
Mesenchymal Stem Cell Chondrogenic Differentiation Medium (Ready-to-use)	100 ml	C-28012
Mesenchymal Stem Cell Chondrogenic Differentiation Medium without Inducers (Ready-to-use)	100 ml	C-28014
Mesenchymal Stem Cell Osteogenic Differentiation Medium (Ready-to-use)	100 ml	C-28013
Mesenchymal Stem Cell Neurogenic Differentiation Medium (Ready-to-use)	100 ml	C-28015
Accutase-Solution, primary human cell culture tested	100 ml	C-41310
Dulbecco's PBS, without Ca^{++}/Mg^{++}	500 ml	C-40232
Cryo-SFM Plus	30 ml	C-29920
	125 ml	C-29922

References

1. da Silva Meirelles L, Caplan AI, Nardi NB., Stem Cells 2008; 26(9):2287–99.
2. Crisan M, Yap S, Casteilla L, et al., Cell Stem Cell 2008; 3(3):301–13.
3. Dominici M, Le Blanc K, Mueller I, Slaper-Cortenbach I, et al., Cytother 2006; 8(4):315–7.
4. Caplan AI., Cell Stem Cell 2008; 3(3):229–30.

PromoCell GmbH
Sickingenstr. 63/65
69126 Heidelberg
Germany

info@promocell.com
www.promocell.com

USA/Canada
Phone: 1 – 866 – 251 – 2860 (toll free)
Fax: 1 – 866 – 827 – 9219 (toll free)

Deutschland
Telefon: 0800 – 776 66 23 (gebührenfrei)
Fax: 0800 – 100 83 06 (gebührenfrei)

France
Téléphone: 0800 – 90 93 32 (ligne verte)
Téléfax: 0800 – 90 27 36 (ligne verte)

United Kingdom
Phone: 0800 96 03 33 (toll free)
Fax: 0800 169 85 54 (toll free)

Other Countries
Phone: +49 6221 – 649 34 0
Fax: +49 6221 – 649 34 40

10/2024