Osteogenic Differentiation and Analysis of MSC

PromoCell

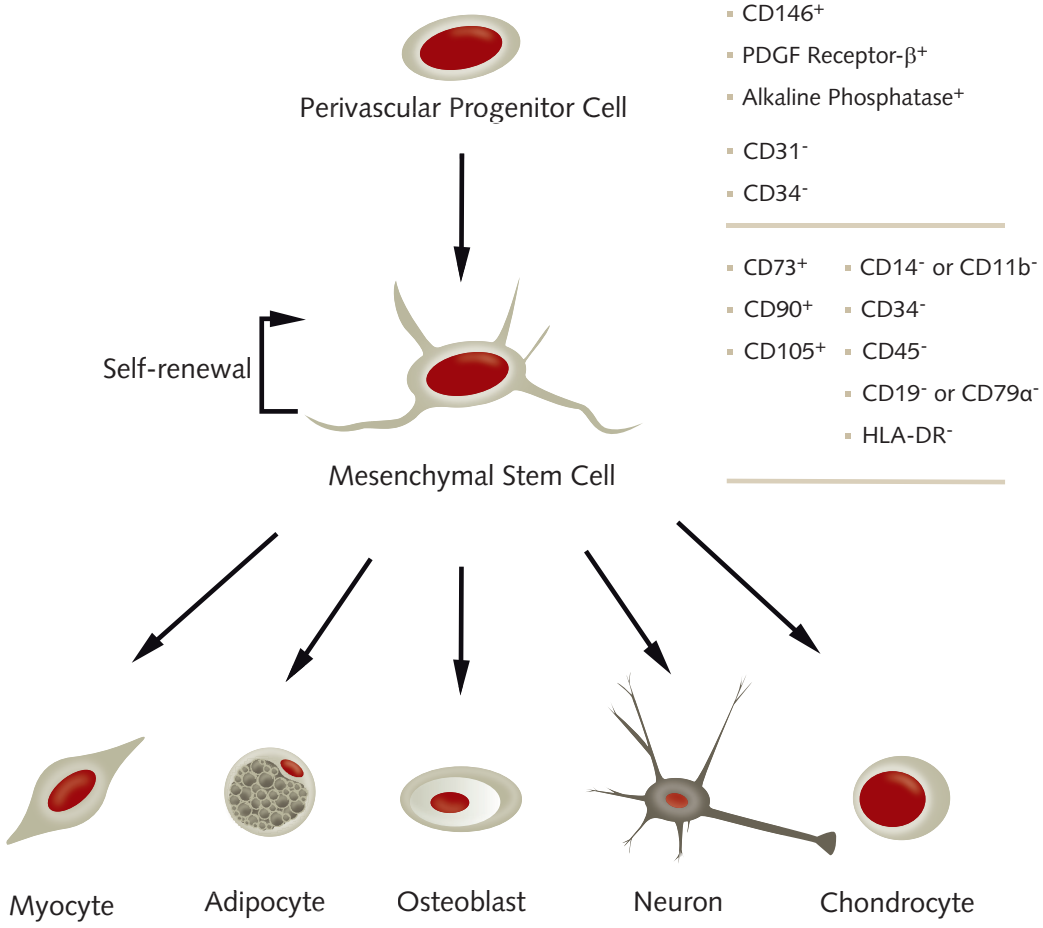
Application Note

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].

Characterization

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]. Trans-differentiation 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 the ready-to-use PromoCell 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

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

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

Use aseptic techniques and a laminar flow bench.

Osteogenic Differentiation

1. Coat the culture vessel
Coat a 6-well tissue culture plate with 10 µg/ml human or bovine fibronectin (C-43060/C-43050) according to the instruction manual.

2. Seed Mesenchymal Stem Cells
Plate MSC at $1x10^5$ cells per well in the fibronectin-coated tissue culture plate using MSC Growth Medium 2 (C-28009). Work in duplicate.

3. Allow Mesenchymal Stem Cells to grow
Important: Allow the cells to reach at least 100% confluency. This will take 48–72 hours.

4. Induce Mesenchymal Stem Cells
Induce one of the duplicate samples with MSC Osteogenic Differentiation Medium (C-28013). Use MSC Growth Medium 2 for the remaining well as a negative control.

5. Induce Mesenchymal Stem Cells
Incubate for 12–14 days. Change the medium every third day. Be careful not to disturb the cell monolayer.

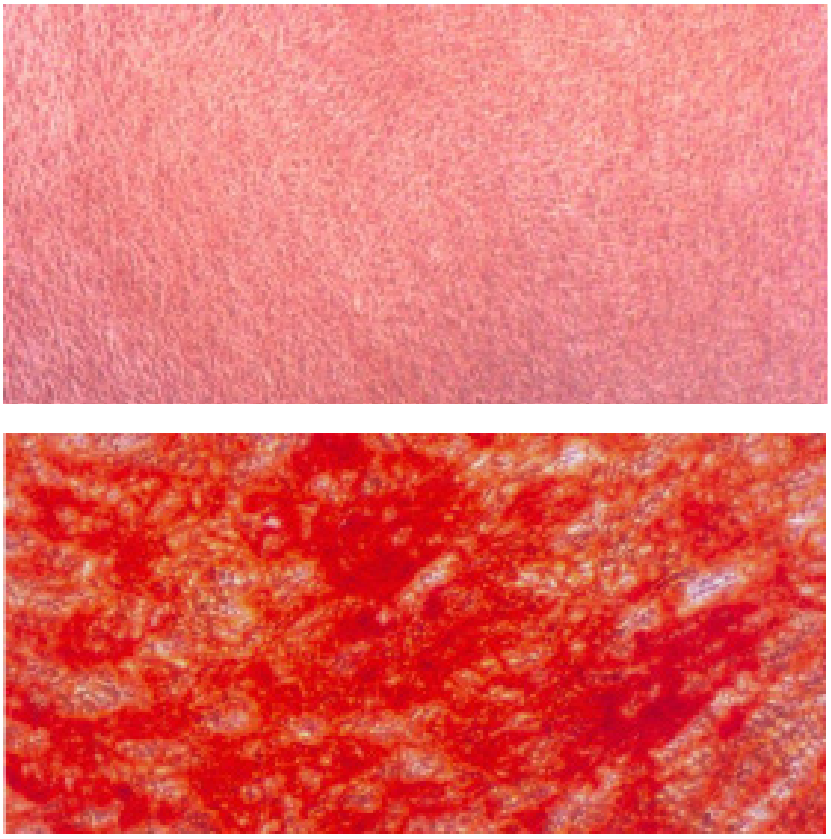


Fig. 1: Alizarin Red S staining of extracellular calcium deposits in mineralized hMSC-BM (human MSC derived from bone marrow)-derived mature osteoblasts. Cells were cultured for 12 days in PromoCell MSC Growth Medium 2 (C-28009) for the negative control (upper panel) or MSC Osteogenic Differentiation Medium (C-28013) for the differentiation sample (lower panel). 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.

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

Osteoblast Detection (Calcium Deposits)

Osteoblast Detection

Please follow the recommended safety precautions for the chemicals used in this 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

Use Saccomanno Fixation Solution (Morphisto, #13881.00250). To prepare the Alizarin Red S 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 the dark-brown solution. Store in the dark at 2–8 °C.

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) w/o Ca^{++}/Mg^{++} (Cat. No. 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 30 min gently aspirate the fixation solution and wash the cells with 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.

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 min. Monitor staining progress for 10 min and stop the process when staining intensity is sufficient.

5. Wash the cells

Carefully aspirate the Alizarin Red S staining solution and wash the cell monolayer four times with 1 ml distilled water. Carefully aspirate the distilled water and add PBS.

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/slightly purple, whereas MSC-derived osteoblasts (with extracellular calcium deposits) stain bright orange-red (Fig. 1).

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

Osteoblast detection (alkaline phosphatase)

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.

1. Prepare solutions and buffers

Dissolve one BCIP/NBT tablet (SigmaFast™ BCIP-NBT; Sigma Aldrich) 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, w/o Ca^{++}/ Mg^{++} (Cat. No. C-40232) 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 of 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.

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 min. Check staining progress every 2–3 min.

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.

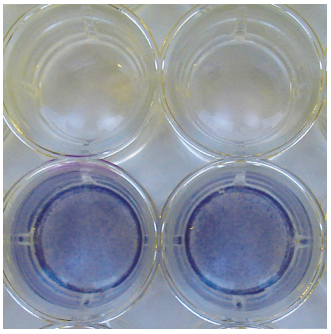


Fig. 2: Undifferentiated MSC (AP negative, upper row) are colorless or faintly bluish, whereas MSC-derived osteoblasts (AP positive, lower row) are dark blue-violet. The higher the AP activity, the more intense the color.

* 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.

Osteoblast Detection

Please follow the recommended safety precautions for the chemicals used in this procedure!

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.

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 DXF (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 w/o 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
Cell Dissociation Solution ACF	100 ml	C-41320
Dulbecco's PBS, w/o Ca^{++}/ Mg^{++}	500 ml	C-40232
Fibronectin Solution, human (1 mg/ml)	5 ml	C-43060
Fibronectin Solution, bovine (1 mg/ml)	5 ml	C-43050
hMSC-BM Pellet	1 million cells per pellet	C-14090
hMSC-UC Pellet	1 million cells per pellet	C-14091
hMSC-AT Pellet	1 million cells per pellet	C-14092

PromoCell GmbH

Sickingenstr. 63/65
69126 Heidelberg
Germany

Email: 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

12/2015