Isolation, culture and, characterization of endometrial mesenchymal stromal cells (endMSCs)

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Method Article

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Abstract

Endometrial Mesenchymal Stromal Cells (endMSCs) can be isolated from menstrual blood of healthy women and expanded under standard culture conditions. Once the cell culture is established, the multipotency of endMSCs can be i*n vitro* evaluated using specific differentiation media for 21 days. Cell differentiation state can be observed by optical microscopy. On the other hand, the phenotypical characterization can be performed by flow cytometry targeting a combination of surface markers.

This protocol is suitable for establishing an endMSCs culture for multiple purposes: *in vitro* assays, preclinical studies, “omic” analyses, etc.

Introduction

The isolation of MSCs from the endometrial decidual tissue in menstrual blood is a non-invasive and reproducible method. This methodology does not require any painful procedure for donors. In terms of laboratory processing, these cells can be selected by plastic adherence and *in vitro* expanded under standard culture conditions. The menstrual blood-derived stromal cells display multipotent capabilities and immunomodulatory effects[1].

The present isolation protocol of endMSCs is based on previous publications[2,3]. Slight modifications have been performed by our group[4,5]. Reagents, equipment, and methodology are described in the cited publications.

Reagents

Reagents:

- Phosphate buffer saline (PBS).
- Trypsin
- Fetal bovine serum (FBS)
- Penicillin-streptomycin
- DMEM
- L-glutamine
- Monoclonal antibodies against CD29, CD31, CD34, CD44, CD45, CD49d, CD49f, CD56, CD73, CD90, CD105, and HLA-DR
- Stem Pro Adipogenesis, Chondrogenesis and Osteogenesis Differentiation Kits (Gibco, Thermo Fisher Scientific, USA)

- Oil Red O Staining
- Alcian Blue 8GX staining
- Alizarin Red S staining

Equipment

- CO_2 incubator
- Laminar flow chamber
- FACScalibur cytometer (BD Biosciences)
- Optical microscope
- Spectrophotometer

Procedure

Isolation and culture of endMSCs:

1- Healthy pre-menopausal women, without infections or immune disorders and who are not undergoing hormonal therapy, can be eligible as potential menstrual fluid donors. Menstrual fluid can be collected by the use of a menstrual cup or in a sterile urine container on day 2 or 3 of the menstrual cycle.

2- Dilute blood samples in PBS. If endometrial tissue fragments are retrieved, mechanical disaggregation is required. Transfer the recovered tissue to a sterile Petri dish and use a scalpel blade to obtain smaller pieces. Add the disaggregated tissue to the diluted blood. Centrifuge at 450 × g for 10 minutes.

3- Discard the supernatants to remove the residues of cervical mucus and resuspend the cells in DMEM containing 10 % fetal bovine serum (FBS), 1% penicillin/streptomycin and 1 % glutamine. Seed the resuspended cells onto tissue culture flasks and incubate at 37 ˚C in 5 % CO_2 atmosphere.

4- After 24 hours, remove non-adherent cells by washing with PBS. Once some colonies are formed, detach the adherent cells using PBS containing 0.25 % trypsin (v/v), seed them at a density of 5 000 cells/cm^2, and expand to 80% confluency.

5- Change culture medium every three days.

Phenotypical characterization:

1- Stain 2 × 10^5 endMSCs with the appropriate concentrations of monoclonal antibodies against CD29, CD31, CD34, CD44, CD45, CD49d, CD49f, CD56, CD73, CD90, CD105, and HLA-DR and incubate for 30 min at 4 ºC in the presence of PBS containing 2% FBS.

2- After incubation, wash the cells and resuspend in PBS.

3- Use isotype-matched antibodies as negative controls.

4- For flow cytometric analyses, acquire 10^5 events on a FACScalibur cytometer (BD Biosciences, CA, USA).

Functional characterization:

1- Culture cells with an 80 % of confluence in a differentiation media for 21 days (Stem Pro Adipogenesis, Chondrogenesis and Osteogenesis Differentiation Kits, Gibco, Thermo Fisher Scientific, MA, USA).

2- After 21 days, fix cells with a paraformaldehyde 4 % solution for 20 minutes.

3- Wash cells 3 times with PBS and stain differentiated cell:

- Adipogenic staining: incubate cells with isopropanol 60 % during 5 min, dry, stain with 3.5 mg/ml de Oil Red O in isopropanol 60 % for 5 min and, wash with distilled water.

- Chondrogenic staining: wash cells twice with HCl 0.1 M, stain with 10 ng/ml Alcian Blue 8GX (diluted in HCl 0.1 M) for 2 h and wash with HCl 0.1 M and distilled water.

- Osteogenic staining: stain with 20 mg/ml Alizarin Red S (pH = 4.1-4.2) for 30 min and wash with distilled water.

4- Quantify cell differentiation by spectrophotometry determining the absorbance of the extracts at 490 nm (Oil Red O and Alizarin Red S staining) and at 600 nm (Alcian Blue 8GX).

5- Confirm the *in vitro* differentiations by microscopic examination at 20X magnification

Troubleshooting

In the first phases of endMSC isolation, just one centrifugation before cell pellets are seeded into culture flasks may not be enough for blood-cell removal (steps 3–4). Discard the supernatants and centrifuge again until the supernatants appear clearer. After 24 hours, multiple washings with PBS may be necessary to completely remove blood cells and observe the plastic adherent-cells under a light microscope.

Culture contamination is the most common problem in the isolation of endMSCs. For this reason, the addition of antifungal supplements, as amphotericin B (Fungizone®) and additional antibiotics, as ciprofloxacin, in the culture medium may prevent bacterial and yeast contamination, especially for the first 10 days.

Time Taken

Time to reach an 80 % of confluence in a culture is variable, depending on the cell division rates. Cells can be isolated 24 hours after seeding but, the expansion will depend on number of cells that is necessary for different protocols.

The phenotypic characterization can be completed in two hours, but differentiation assays and staining take 21 days.

The estimated times do not include the analysis of results.

Anticipated Results

The phenotypic analysis, demonstrated that endMSCs were negative for CD14, CD20, CD34, CD45, CD80, HLA-DR and positive for the stemness markers CD44, CD73, CD90 and CD117. Figure 1A shows the representative histograms for these markers and the numbers within the histograms represent the Mean Relative Fluorescence Intensity (MRFI) and standard deviations from different donors (n = 4). It is interesting to note that we did not observe any significant change in the phenotype of endMSCs after different passages and their phenotypes were very similar when different donors were compared.

In order to demonstrate the multipotency of isolated and *in vitro* expanded endMSCs, the adipogenic, chondrogenic and osteogenic differentiation potential was evaluated according to standard differentiation protocols. The endMSCs were cultured for 21 days and differentiation was evaluated with Alizarin Red, Alcian Blue and Oil Red O stainings. The stains were solubilized and the resulting absorbances were quantified by spectrophotometry (Figure 1B). As shown in the representative images, the differentiation towards adipogenic, chondrogenic and osteogenic lineages was confirmed by microscopy (Figure 1C).

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Figures

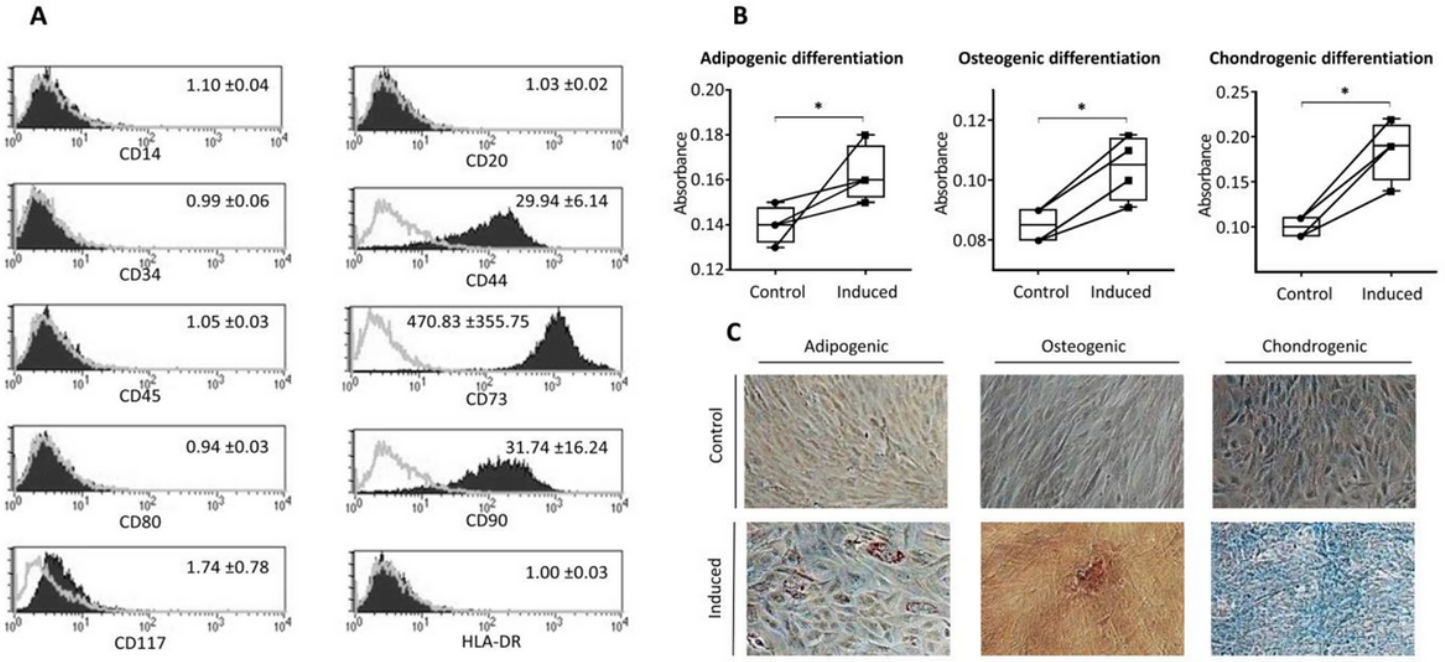

Figure 1

Phenotypic analysis of endMSCs and multipotent differentiation assays. A) Phenotypic analysis was performed by multicolor flow cytometry. Representative histograms of four different cell lines are shown. The expression level of cell surface markers (CD14, CD20, CD34, CD44, CD45, CD73, CD80, CD90, CD117 and HLA-DR) is represented as Normalized Mean Relative Fluorescence Intensity (MRFI) which is calculated by dividing the Mean Fluorescent Intensity by its isotype control. The MRFI values and the

standard deviations obtained from four different cell lines are included in the upper right corner of the histograms. B) Adipogenic, osteogenic and chondrogenic differentiation of endMSCs were induced and quantified by determining the absorbance of the extracts at 490 nm (Oil Red O and Alizarin Red S staining) and at 600 nm (Alcian Blue 8GX). Four independent experiments using four different cell lines were performed and a Mann-Whitney U test was used. C) The in vitro differentiations were confirmed by microscopic examination at 20X magnification. The upper images correspond to control cells (non-induced) and lower images to in vitro induced differentiations