# HUMAN MESENCHYMAL STEM CELLS AS BASIC TOOLS FOR TISSUE ENGINEERING: ISOLATION AND CULTURE

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*Abstract*. Stem cells are immature cells capable of autoreplication, which are able to generate various mature cell types and have a remarkable viability and proliferative capacity. Mesenchymal stem cells (MSC), under proper conditions, can give rise to osteoblasts, adipocytes, chondrocytes, myocytes and even neurons. Several procedures applied in tissue engineering imply harvesting autologous MSC, expanding them in culture without loss of stemness, inducing differentiation, seeding them on suitable scaffolds in accordance with the targeted tissue type and implanting the construct into the patient's body. During this study, bone marrow has been extracted from 9 patients by aspiration from the upper posterior iliac crest and used in order to isolate the MSC by employing three different methods: (1) the Ficoll-Paque technique for the isolation of mononucleated cells followed by the separation of MSC by adherence to plastic, (2) Ficoll-Paque followed by the immuno-magnetic separation of MSC using a CliniMACS system and (3) a negative selection procedure of MSC using the RosetteSep technique followed by adherence to plastic. We have prepared and optimized our media, assuring control and reproducibility of the results. Finally, an immunophenotypic characterization of the cells by flowcytometry has been performed. On the 3$^{rd}$ day after seeding the isolated mononucleated fraction in the optimally prepared culture medium, we observed elongated adherent cells which have undergone extensive proliferation between days 5 and 9, with colony forming around days 10 to 14. The cell population reached 70 – 80% confluence within 3 weeks in culture. Independent on the isolation procedure, the largest number of cell colonies has been obtained for a cell seeding density of $10^6$ cells/cm$^2$.

*Key words*: stem cells, differentiation, plasticity, flowcytometry.

## INTRODUCTION

In the case of tissue damage, transplant is one of the very few clinical solutions available today. Nevertheless, its feasibility is limited by donor organ shortage. In an effort to circumvent this problem, about two decades ago a new field of medical sciences emerged, which goes by the name of tissue engineering (TE) and aims to develop biologically based replacement tissues. Several problems must be solved before TE is used on large scales in clinical practice. These include

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questions regarding a suitable cell source, the identification of the appropriate scaffold for each tissue type of interest and the optimization of the culture conditions in order to ensure viability and physico-chemical properties comparable to the native organ.

Stem cells (SC) are immature cells with two essential features: (1) they are capable of autoreplication and (2) they are able to give rise to various mature cell types of the organism. In culture, they have a remarkable viability and proliferative capacity [1, 2]. All these characteristics make them a favourite cell source for TE.

The bone marrow is regarded as the most important non-hemopoietic, mesenchymal stem cell (MSC) source. MSCs have been identified in the periosteum, muscle, liver, blood and fetal bone marrow, whereas their occurrence in the umbilical cord blood is controversial. By supplementing the cell culture medium with specific growth and differentiation factors, MSC may be coaxed to give rise to osteoblasts, chondrocytes, myocytes, adipocytes, cardiomyocytes and neurons, too [7].

In order to use MSC in regenerative medicine, it is necessary to isolate and proliferate them without loss of stemness, i.e. preserving their pluripotentiality and proliferative capacity in undifferentiated state. Recent studies are focused on developing optimal culture conditions (including cell culture media, bioactive agents and biodegradable scaffolds) for each specific therapy [9].

## MATERIALS AND METHODS

### PATIENTS

Bone marrow from 9 patients (10 ml/patient) has been extracted from the upper posterior iliac crest using a heparinated syringe, under general anesthesia, in a sterile surgical room, in accordance with ethical regulations and having the acceptance of the patients.

### CELL SEPARATION

We employed three methods of isolation of MSC from bone marrow: (1) the Ficoll-Paque technique for the isolation of mononucleated cells followed by the separation of MSC by adherence to plastic, (2) Ficoll-Paque followed by the immuno-magnetic separation of MSC and (3) a negative selection procedure of MSC using the RosetteSep technique, followed by adherence to plastic.

1. The Ficoll-Paque technique of density gradient centrifugation was employed by first diluting the bone marrow sample with cell culture medium supplemented with 2% PBS and 1–2 mM EDTA; the resulting solution was poured on the top of the separation medium (Ficoll-Paque solution of 1.077 g/ml density), in a 50 ml centrifuge tube (Becton Dickinson, USA) and centrifuged at room

temperature, at 445×*g*, for 35 minutes. The majority of the mononucleated cells accumulated on the Ficoll-plasma interface. The centrifugation was repeated for 10 minutes at 300×*g*. Finally, the cells were resuspended and counted using a hemocytometer.

2. RosetteSep combines the density gradient separation with antibody-mediated specificity. The bone marrow was placed into a centrifuge tube (Becton Dickinson, USA) and, after adding the RosetteSep solution, the mixture was incubated at room temperature for 20 minutes and centrifuged for 25 minutes at 300×*g*. The result was a negative selection of MSC, which have not been targeted by antibodies and accumulated on the plasma-Ficoll interface. The mononuclear cell suspension obtained by Ficoll or RosetteSep separation technique was plated on Petri dishes for selective isolation by adhesion. The cells were incubated at $37^{o}$C, in 5% $CO_2$ and 95% minimal humidity at different seeding densities: 5, 10, 15, 50, 100 and 500 x $10^4$ cells/$cm^2$ . The non-adherent cells were eliminated on the $3^{rd}$ day after seeding by replaceing the culture medium. The adherent cells were cultured until 70 – 80% confluence was attained (14–21 days) with medium change every $4^{th}$ day.

3. Isolation using MACS – Direct CD105 consists in magnetic sorting of CD105+ cells. To this end, we used a MidiMACS Separator (Miltenyi Biotec, Germany). The cells were immunomagnetically labeled using a cell-type specific reactive. The suspension obtained by the Ficoll technique was mixed with MACS CD105 superparamagnetic colloidal MicroBeads, (Miltenyi Biotec, Germany) coated with CD105 monoclonal antibodies: and incubated for 15 minutes at 6-12°C. The magnetically labeled separated cells were used in part for flowcytometric and viability analyses, the rest being cultured.

## CELL CULTURE

We used Dulbecco's Modified Eagle's Medium (DMEM) low glucose (Sigma-Aldrich, Germany), supplemented with 10% Fetal Calf Serum (FCS) (Promocell, Germany), 2 ng/ml FGFb (Sigma-Aldrich, Germany) and 1% antibiotic/antimycotic (penicillin 10.000 u/ml; streptomycin 10 mg/ml; amfoterycin B 0,25 μg/ml) (Sigma-Aldrich, Germany). The cells were plated on T25 culture dishes (Becton Dickinson, USA) at 6 values of the seeding density, and incubated at 37 °C in an atmosphere of 95% relative humidity and 5% $CO_2$. The first medium change was done 72 h after seeding, then medium was changed weekly.

For passage, the medium was discarded, the system was washed with PBS, cells were detached using trypsin/EDTA 0,25%. After trypsin neutralization with DMEM supplemented with 10% FBS, the cell suspension was centrifuged at room temperature for 10 minutes at 300×*g*, the supernatant was removed and the cells were resuspended, counted and distributed in culture dishes.

FLOWCYTOMETRIC ANALYSIS

The cells have been phenotypically characterized by using a flowcytometer (FACSCalibur, Beckton Dickinson). After trypsinization, 100.000 cells were incubated with fluorescence-conjugated antibodies (marked with FITC–fluorescein isothiocyanate and PE–phycoerythrin fluorochromes, Beckton Dickinson) for 20 min in the dark. After 2 washing steps with PBS, cells were acquired in FACSCalibur (Becton Dickinson) flowcytometer using CellQuest software and analysed with Paint-A-Gate software.

## RESULTS

We harvested human bone marrow from 9 patients, made a comparative study of three isolation techniques, cultivated and analysed the MSC and performed a morphological characterization together with a flowcytometric analysis of the cells.

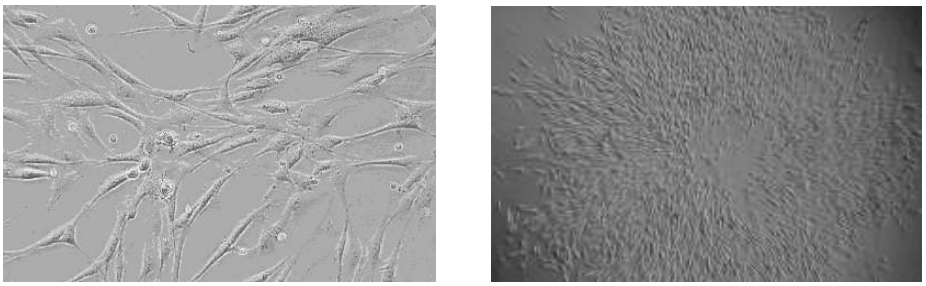

Fig. 1. Mesenchymal stem cells on day 14 (left, 200x magnification), and on day 21 after seeding, respectively (right, 40x magnification).

On the 3$^{rd}$ day after seeding we observed elongated adherent cells; these have undergone extensive proliferation between days 5 and 9, with colony formation around day 10 to 14. The cell population reached confluence within 3 weeks in culture. During long-term cultivation, cells were passed after 2 weeks from seeding, at 70–80% confluence, and were examined during 8 weeks.

The initial flowcytometric analysis, performed on day 0 when cells were seeded, displayed a heterogeneous mixture of cells with different forward and sideward scattering profiles (data not shown). A major proportion of the cells expressed CD45 and Glycophorin A, while the specific MSC markers, CD105 and CD73 were not expressed. The immunophenotype of the cells resulted from passage 1 (day 21) showed low levels of CD14, Glycophorin A and CD45, probably due to contaminating cells (data not shown). The flowcytometric analysis revealed a significant increase of CD 73 expression at the second passage (day 42).

The hemopoietic stem cell marker, CD34, was not expressed neither on day 0, nor after the two passages. The data have been displayed in the form of single-colour histograms, evidencing the CD34, CD45, CD90, CD105 and CD73 expression by the investigated cells (Fig. 2).

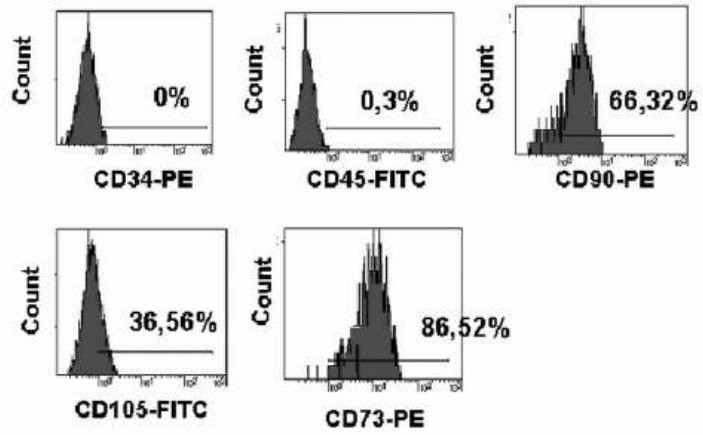


Fig. 2. Immunophenotypic characterization of the cells by FACS analysis, on day 42 after seeding (passage 2). Each plot is a histogram depicting the number of cells vs. their fluorescence level. The numbers specify the percentage of cells expressing the specific markers (with fluorescence falling between the marker boundaries, marked by the horizontal segments).

## DISCUSSIONS

During the past two decades MSC have been extensively investigated because of their far–reaching therapeutic potential [10, 11]. From the point of view of cell and gene therapies, they display several exciting properties such as ease of isolation, good proliferative capacity and ease of transfection with exogeneous genes. Several procedures proposed for tissue repair imply harvesting autologous mesenchymal stem cells, expanding them in culture, inducing differentiation, seeding them on suitable scaffolds in accordance with the targeted tissue type and implanting the construct into the patient's body [4, 6, 8].

The composition of the cell culture medium turned out to be a decisive factor in preserving the stem cell features [5, 9]. Under certain conditions MSC may be passed 25–30 times without observing signs of spontaneous differentiation. Yet other conditions coax MSC to engage towards one of several cell lineages; this property goes by the name of stem cell plasticity [3, 7].

Human MSC isolated in our laboratories have expressed typical cell surface markers at levels in good agreement with recent results of similar studies. A typical MSC marker, CD105, displayed a slight regression from day 21 to day 42, presumably due to spontaneous differentiation of the cells, while the expression of CD90 and CD73 showed a significant increase. However, even after 42 days, 36,56% of the cells express CD105, 86,52% express CD73 and 66,32% express CD90 suggesting that their stemness has been preserved.

By comparing the mean number of formed colonies, counted during all 21 performed experiments, no significant differences in colony formation between the three distinct MSC isolation methods have been observed (data not shown). This comparison allowed us to find the optimal cell seeding density ($10^6$ cells/$cm^2$) which assured a maximal number of colonies.

These observations enable us to conclude that the cell culture media and conditions were suitable for MSC expansion. Future work will be focused on human MSC differentiation in vitro towards the osteogenic and chondrogenic lineages.

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