

A novel stem cell tag-less sorting method

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ABSTRACT

The lack of homogeneity in stem cell preparation blurs standardization, which is recommended for successful applications. Since common procedures for stem cell sorting involve approaches which make use of fluorescent or magnetic tags which can be not precisely known or can affect cell functionality and recovery, novel, fast and non-invasive methods for cell sorting are sought. Among stem cells, mesenchymal stem cells (MSCs) are promising candidates for cell therapy applications. This work presents a fractionation protocol based on a proprietary technology for the tag-less, flow-assisted fractionation of MSCs. The process is based on differences in cell biophysical features and it guarantees minimal manipulation and maintenance of cell features. It can be applied to isolate and separate MSCs from potential phenotypically different contaminants; to distinguish MSCs derived from different sources, and to sort stem cells from an MSC population isolated from a single source to improve the differentiation yield.

Keywords: tag-less cell sorting, stem cell, cell fractionation, mesenchymal stem cell, flow-assisted cell separation

1 INTRODUCTION

Stem cells are distributed in all tissues and are particularly available from sources like bone marrow, dental pulp, adipose tissue, peripheral blood, umbilical cord and fetal membrane. Stem cells which are phenotypically different from each other are commonly sorted using approaches based on flow cytometry (FC) which makes use of fluorescent or magnetic tags (FACS, fluorescent-activated cell sorting and MACS, magnetic-activated cell sorting). However it is widely recognized that the expression of surface antigens of stem cells is rich and diversified to make their immuno-phenotypical characterization not precise and easy [1]. On the other hand, immuno-tags for cell identification are known to affect cell

functionality and recovery [2]. Thus, novel, fast and non-invasive methods that are not based on cell immuno-tagging are sought to improve stem cell sorting.

Field-flow fractionation (FFF) is a separative flow-assisted method able to fractionate analytes with high molar mass, among which cells. In FFF, living cells are fractionated via morphological and biophysical differences within cellular populations with maintenance of cell features and viability. FFF is achieved within an empty capillary channel by the combined action of a transporting laminar flow of biocompatible fluid and a field applied perpendicular to the flow. Depending on the nature of the applied field, different variant of FFF have been developed with peculiar applications ranging from analysis of analytes with interest in nanotechnology, proteomics and the development of bioanalytical assays [3]. The simplest variant of FFF makes use of Earth's gravity field (Gravitational field-flow fractionation, GrFFF) to obtain cell fractionation. Due to its high simplicity and biocompatibility, GrFFF allows the fractionation of cells under easy sterilization conditions and with a disposable device. An application of GrFFF to sort cells from complex matrices concerns the enrichment in hemopoietic stem cell from leukapheresis samples [4].

Among human adult stem cells, mesenchymal stem cells (hMSCs) are considered promising candidates for clinical applications based on cell-therapy approaches. They are adherent, multipotent stem cells that can be isolated from various connective tissues [5, 6] such as bone marrow (BM), fetal membranes (FM), adipose tissue (AT) and dental pulp (DP). hMSCs have an immunomodulatory function and potentially inhibit the immune response. However, hMSCs from various sources exhibit differing lineage-commitment yields and differing expression levels of pluripotency markers, very likely because of the presence of dissimilar progenitor cells [5].

As reported in previous literature, phenotypically similar hMSCs deriving from different sources show differences in their ultrastructural characteristics which can be related to dissimilar differentiation abilities [7].

Transmission electron microscopy (TEM) has been used to investigate the basic ultrastructural phenotype of MSCs. The study revealed subtle yet significant differences among hMSCs isolated from different tissues which could be exploited for a separation based on GrFFF. However, application of the conventional GrFFF method to adherent cells requires cell sedimentation at the accumulation wall, which tends to cause cell adhesion to the wall and cell-cell aggregation/stacking with a reduction of recovery and cell functionality. As a consequence, a variation of the GrFFF approach was proposed for the development of a protocol suitable for the biophysical fractionation of adherent stem cells. The fractionation protocol is based on non-equilibrium GrFFF as a means of purifying, distinguishing and sorting MSCs from clinical specimens [8, 9].

Cell contact and adhesion with the fractionation device are avoided by in-flow injection, by the absence of stop-flow cell sedimentation, and by using elution flow rate values able to generate hydrodynamic forces that are intense enough to lift and keep cells away from the channel wall. Cells can be separated directly from the crude sample; cells are suspended in a fluidic condition and the fractionation process is based on the differences in cell features that are dynamically acquired during flow-assisted fractionation under the combined action of the flow stream, the gravitational field, and the hydrodynamic lift forces.

The flow rate values applied guarantee low shear stress on cells. After fractionation is completed, cells can return to the adherent state, and the native physical features are fully restored. Cell viability after fractionation proves to be fully preserved upon using standard flow-cytometry (FC)-based staining methods.

The protocol can be applied to separating MSCs from potential phenotypically different contaminants when cells are isolated from clinical specimens, thus allowing the reduction of cell culture passages for a precise selection allowing for the maintenance of native cell properties; to distinguish MSCs derived from different sources, and finally to sort stem cells from an MSC population isolated from a single source, obtaining an high improvement of differentiation yield. Moreover, the protocol results simple to use and integrate in conventional cell-sorting platforms to reduce time and improve fully functional stem cell yield.

2 MATERIALS AND METHODS

2.1. Isolation and characterization of hMSCs from different sources and amniotic epithelial cells

Upon informed consent, hMSCs were isolated from adult sources like bone marrow (BM), fetal membranes (FM), amniotic membrane (AM) obtained from term placenta, dental pulp (DP) and adipose tissue (AT). The isolation procedures followed were specific for different cell sources, and took advantage of the MSC property of

adhering to culture flasks [5]. Amniotic Epithelial Cells (AECs) were isolated from fetal membranes as previously described [5]. Cell populations were subsequently characterized by flow cytometry using a panel of well-established mesenchymal and epithelial markers. The antibodies used were: anti-CD29-FITC, anti-CD34-PE, anti-CD44-FITC, anti-CD45-APC, anti-CD90-PC5, anti-CD105-PE, anti-CD166-PE (all from Beckman Coulter, Fullerton, CA, USA), anti CD73-FITC (BD-Pharmingen, San Diego, CA, USA) and anti-pan-Cytokeratin (PanCK C11-PE, Santa Cruz Biotechnology, USA). Samples were analyzed using a Cytomics FC500 flow cytometer equipped with two lasers (Beckman Coulter). Results were analyzed using CXP Software (Beckman Coulter). The hMSCs were then tested for typical mesenchymal differentiation potentials (adipogenic, osteogenic, and chondrogenic) in accordance with established procedures [5].

2.2. Fractionation protocol

The fractionation device was a ribbon-like capillary channel where two polyvinylchloride walls sandwiched a thin foil of polyethylene terephthalate from which the channel volume had been removed. Channel dimensions were 2.0 cm in breadth, 0.014 cm in thickness and 30 cm in length.

The cells were injected into the fractionation device by means of a continuous flow delivered by a peristaltic pump (Miniplus 3, Gilson, Middleton, WI, USA). The flow rate was set at 0.46 ml/min. Sterile phosphate buffer saline (PBS) mixed with 0.1% BSA, 100 U/ml penicillin, and 100 µg/ml streptomycin was employed as the transport fluid. At the fractionation device outlet, a UV/Vis detection system was connected to monitor the elution process. A fraction collector was connected downstream of the detector outlet to collect the eluted cells. The system was placed in a laminar-flow hood to assess sterile conditions. Decontamination of the fractionation system was performed at the beginning of each working day by flushing sodium hypochlorite in sterile water at 2% as active chlorine.

Cells were counted and resuspended in the mobile phase at a concentration of 3×10^6 cells/ml. Cells had to be properly maintained in suspension to avoid cell aggregation. As a rule, 45 µL of cell suspension was injected into the fractionation device by means of an injection valve. After a relatively short period of time from injection (about 30 minutes), cell elution was complete.

When necessary, eluted cells were collected at the fractionation device outlet as selected fractions. After fractionation, total cell recovery was always achieved. In each fraction the cell amount was at least 10^4 cells. Repeatability of the fractionation process was always high (run-to-run %CV values of the retention times below 3%). Cells collected at the same elution time values from three repeated runs were pooled and subjected to characterization, expansion and/or commitment to a specific differentiation, or stored for further use.

3 TAG-LESS FRACTIONATION OF MSCs

This protocol was used for the: purification of fetal membrane-derived hMSCs from contamination consisting of AECs; characterization of MSCs from different sources; sorting of a MSC population prepared from a single source.

3.1. Purification of fetal membrane-derived h-MSCs

In order to verify the ability of the protocol to purify stem cells from contaminants in an early stage of their isolation, the example of MSCs isolated from human placenta where AECs represent an important source of undesider cells were considered. Heterogeneous population of MSCs and AECs were prepared and injected and fractionated following the fractionation protocol described above. A representative fractogram formed of a baseline separation of two major bands was obtained, as reported in Figure 1. Cell fractions were collected for each of the two bands: F1, from 3 min to 5 min and F2 from 11 min to 13.5 min. F1 and F2 were subjected to microscopic and flow cytometric characterization.

Figure 1 shows as F1 cells displayed the classic fibroblast-like morphology of hMSCs; while, F2 cells showed cobblestone-shaped cells.

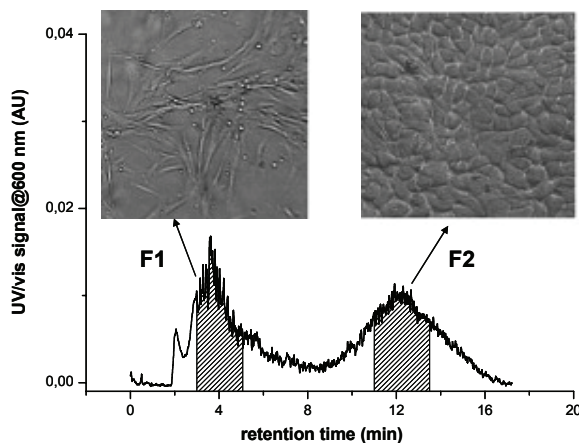


Figure 1. fractogram obtained for a mixture of AECs and FM-hMSCs with F1 and F2 as collected fractions.

The characterization of immunophenotype was completed by flow cytometric analysis: F1 cells confirmed the expression of classic mesenchymal antigens (CD44^{high} and CD105^{high}), and the absence of epithelial markers (i.e. cytokeratins, recognized through mAb pan-CK). F2 cells, by contrast, showed positive cytokeratin expression (PanCK⁺), and a low level of CD44 and CD105. These results are consistent with the observed morphology.

These results confirm that the protocol can be applied to obtain purified MSCs cells from clinical specimens without a high number of cell culture passages.

3.2. Characterization of stem cells derived from various sources

hMSCs from various sources and AECs were fractionated by means of the fractionation protocol. Highly-reproducible fractograms were obtained that were characteristic for each source and could be exploited to characterized phenotypically different MSCs. Results are shown in Figure 2.

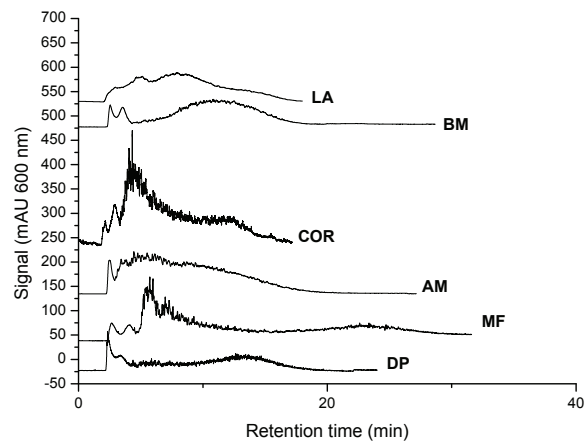


Figure 2. Charatcteristic fractograms obtained for hMSCs isolated from different human sources. (LA: lipoaspirate; BM: Bone Marrow; AM: Amniotic Membrane; MF: Fetal Membrane; DP: Dental Pulp).

These different profiles can be correlated with the different differentiation potential of such cells with an improvement of sorting of stem cells which is fundamental for their application to clinical field.

3.3. Sorting of hMSCs from a single source

In order to evaluate the ability of the protocol to select stem cells with well defined properties among an population isolated from a specific source, hMSCs obtained from adipose tissue were fractionated. By the fractionation protocol, a well-defined fractographic profile was obtained and four fractions (F1, F2, F3, F4) were collected at the following interval time: F1, from 3 min to 5 min; F2, from 6 min to 10 min; F3, from 11 min to 15 min; F4, from 16 min to 18 min.

F1-F4 were subjected to adipogenic differentiation: cells from each fraction and an unfractionated control (UC) were seeded in 6-well plates at a density of 17×10^3 cells/cm². Finally, the differentiation yield of the subpopulations was

investigated by microscope after Red Oil staining. Results are reported in Figure 3.

Significant differences in differentiation yield were evaluated: F1=10%, F2=95%, - F3=30% F4=10%; and UC=40%. Cells in F2 (the fraction collected at the band maximum) showed the highest commitment yield even compared to non fractionated cells, with differentiation efficiency close to 100%. This means that F2 was about 60% richer in differentiating progenitors than UC.

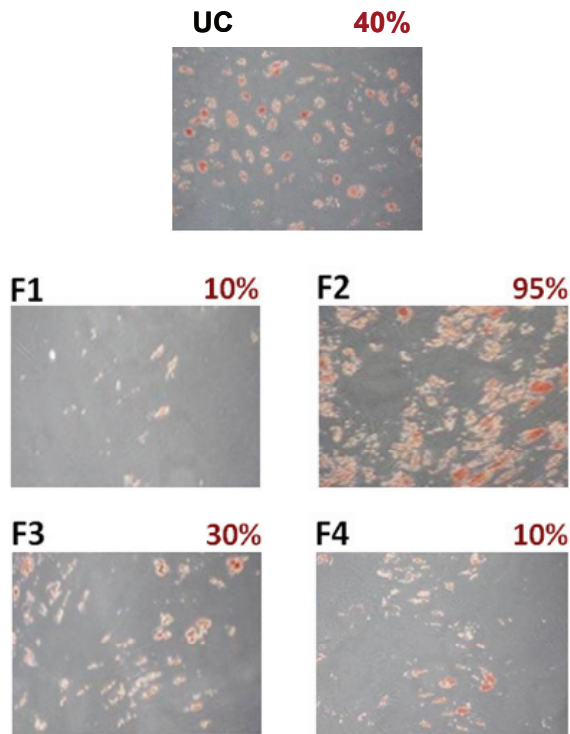


Figure 3. Microscope observation after Red Oil staining of F1-F4 from the fractionation of hMSCs from lipoaspirate, after induction toward adipogenic differentiation. Sorted subpopulations displayed major differences in their adipogenic differentiation yield (%).

These results confirm the ability of the proposed protocol to select well defined stem cells among a cell population isolated from a single source open interesting perspectives in the use of the protocol in addition to cell culture manipulation to drastically improve potentialities of selected cells.

REFERENCES

- [1] Tong J, Hoffman R, Siena S, Srour EF, Bregini M, Gianni AM., *Exp Hematol* 1994; 22: 1016-1024.
- [2] Flanagan LA, Lu J, Wang L, Marchenko SA, Jeon NL, Lee AP, Monuki ES., *Stem Cells* 2008; 26: 656-665.
- [3] Reschiglian P, Zattoni A, Roda B, Michelini E, Roda A, *Trends Biotechnol.* 23 (2005) 475-483.
- [4] Roda B, Reschiglian P, Alviano F, Lanzoni G, Bagnara GP, Ricci F, Buzzi M, Tazzari PL, Pagliaro P, Michelini E, Roda A, *J Chromatogr A.* 2009;1216(52):9081-7.
- [5] Parolini O, Alviano F, Bagnara GP, Bilic G, Bühring HJ, Evangelista M, Hennerbichler S, Liu B, Magatti M, Mao N, Miki T, Marongiu F, Nakajima H, Nikaido T, Portmann-Lanz CB, Sankar V, Soncini M, Stadler G, Surbek D, Takahashi TA, Redl H, Sakuragawa N, Wolbank S, Zeisberger S, Zisch A, Strom SC., *International Workshop on Placenta Derived Stem Cells. Stem Cells* 2008; 26: 300-11.
- [6] Pierdomenico L, Bonsi L, Calvitti M, Rondelli D, Arpinati M, Chirumbolo G, Becchetti E, Marchionni C, Alviano F, Fossati V, Staffolani N, Franchina M, Grossi A, Bagnara GP, *Transplantation* 2005; 80: 836-42.
- [7] Pasquinelli G, Tazzari P, Ricci F, Vaselli C, Buzzi M, Conte R, Orrico C, Foroni L, Stella A, Alviano F, Bagnara GP, Lucarelli E, *Ultrastruct Pathol* 2007; 31: 23-31.
- [8] Reschiglian P, Roda B, Zattoni A, Bagnara GP, *Method and device to fractionate stem cell. Patent pending PCT/EP2007/054226.*
- [9] Roda B, Lanzoni G, Alviano F, Zattoni A, Costa R, Di Carlo A, Marchionni C, Franchina M, Ricci F, Tazzari PL, Pagliaro P, Scalinci SZ, Bonsi L, Reschiglian P, Bagnara GP, *Stem Cell Rev.* 2009 Dec;5(4):420-7.