

A Novel Stem Cell Tag-Less Sorting Method

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Abstract Growing interest in stem cell research has led to the development of a number of new methods for isolation. The lack of homogeneity in stem cell preparation blurs standardization, which however is recommended for successful applications. Among stem cells, mesenchymal stem

cells (MSCs) are promising candidates for cell therapy applications. This paper presents a fractionation protocol based on a tag-less, flow-assisted method of purifying, distinguishing and sorting MSCs. The protocol entails a suspension of cells in a transport fluid being injected into a ribbon-like capillary device by continuous flow. In a relatively short time (about 30 min) sorted cells are collected. The protocol has been applied to the improvement of MSC isolation, with a specific view to reducing cell manipulation operations, keeping instrumental simplicity and increasing analytical information for cell characterization. Applications such as MSC purification from epithelial contaminants, MSC characterization from various human sources and sorting of MSC subpopulations with high differentiation potential are described. The low cost, full biocompatibility and scale-up potential of the protocol presented could make the procedure attractive for stem cell selection.

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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. Due to the fact that stem cells are scantily present in post-natal human tissues, effective methods of cell sorting/enrichment are required.

Common procedures for stem cell sorting involve flow cytometry (FC) which makes use of fluorescent or magnetic tags (FACS, fluorescent-activated cell sorting and MACS, magnetic-activated cell sorting) to isolate and separate cells which are phenotypically different from each other. However,

stem cells express a rich and diversified panel of surface antigens. It is widely recognized that this fact makes characterization of stem cells (SC) less precise and easy on an immuno-phenotypical basis: there is no specific or well-defined stem cell marker; cell-type-specific markers are limited and often recognize multiple members of an SC lineage [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 method able to fractionate living cells via morphological and biophysical differences within cellular populations [3]; cell properties and function are maintained after fractionation. FFF is achieved within an empty capillary channel by the combined action of a transporting laminar flow of biocompatible fluid and a field that is applied perpendicularly to the flow [4]. The simplest variant of FFF makes use of Earth's gravity field (Gravitational field-flow fractionation, GrFFF) to obtain cell fractionation [5,6]. Due to its high simplicity and biocompatibility, GrFFF poses no technical issues for the fractionation of cells under easy sterilization conditions and the fractionation device, once used, may be disposed of. GrFFF has been used to sort different human stem cells and to enrich leukapheresis samples from healthy human donors [7].

Human 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 [8–13] such as bone marrow (BM), fetal membranes (FM), adipose tissue (AT) and dental pulp (DP). MSCs have an immunomodulatory function and potently inhibit the immune response [14]. Pluripotent hMSC subpopulations, able to differentiate into lineage cells other than mesenchymal lineage cells, have also been found [15]. 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 [8,11,16,17].

As reported in previous literature, phenotypically similar hMSCs deriving from different sources show differences in their ultrastructural characteristics [18] which can be related to dissimilar differentiation abilities [11,16,17]. 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. Hence, upon fractionation by GrFFF, there may be differences in the retention behavior of hMSCs from various sources, and these might be associated with differences in their differentiation potential. 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. This can drastically reduce cell recovery, and also affects cell functionality after fractionation.

This paper describes a fractionation protocol based on non-equilibrium GrFFF as a means of purifying, distinguishing and sorting MSCs from clinical specimens [19,20].

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. Since during fractionation cells are suspended in a fluidic condition, they acquire features that may be different from their native, adherent state. The fractionation process is therefore 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 to reduce the number of cell culture passages for MSC selection; to distinguish MSCs derived from different sources, and finally to sort stem cells from an MSC population isolated from a single source, obtaining the highest differentiation yield.

The protocol represents a new tool for tag-less stem cell purification and sorting which can be easily integrated in conventional cell-sorting platforms to reduce time and improve fully functional stem cell yield.

Materials

Isolation and Culture of Mesenchymal Stem Cells (MSCs) and Amniotic Epithelial Cells (AECs)

1. Sterile Phosphate buffered saline (PBS, Lonza)
2. Penicillin-Streptomycin solution (Sigma)
3. Dulbecco's modified Eagle's medium (DMEM, Lonza)
4. Fetal bovine serum (FBS, Lonza)
5. 0.25% Trypsin-EDTA solution (Lonza)
6. Collagenase 1 mg/mL (Sigma)
7. Ficoll (Sigma)
8. Scalpels
9. Tweezers
10. Cell culture flasks (BD Falcon)

11. Epidermal growth factor (EGF, Sigma)
12. Incubator

Characterization of MSCs and AECs

1. Cytomics FC500 flow cytometer (Beckman-Coulter)
2. CXP Software (Beckman-Coulter)
3. Antibodies: 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), anti-pan-Cytokeratin (PanCK C11-PE, Santa Cruz Biotechnology, USA), fluorochrome-matched isotype control irrelevant antibodies (Beckman Coulter, Fullerton, CA, USA).

Adipogenic Differentiation of MSCs

1. Adipogenic Differentiation Medium BulletKit (Lonza Walkersville Inc)
2. Cell culture flasks (BD Falcon)
3. Incubator
4. Red Oil solution

Tissue Collection

All tissue samples were obtained after informed consent, in accordance with the policy approved by the local ethical committee, transferred to the laboratory and used immediately.

Solutions for Fractionation System

Sterilization solution:

To prepare sterilization solution: add to 200 ml of ultrapure water, 200 mL of sodium hypochlorite solution reagent grade available chlorine $\geq 4\%$ (Sigma).

Sterile mobile phase:

To prepare phosphate buffer saline (PBS): dissolve in 1 l of ultrapure sterile water 0.2 g KCl, 0.2 g KH_2PO_4 , 8 g NaCl, 1.43 g $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$. To prepare *complete mobile phase*, add to PBS, 1 g of bovine serum albumin (BSA) (Sigma), pass on a $0.2 \mu\text{m}$ -sterile filter and add 1000 U/ml penicillin and 1000 $\mu\text{g/ml}$ streptomycin.

Fractionation System

Fractionation System Set-Up

The fractionation device was a ribbon-like capillary channel comprised of two polyvinylchloride walls sandwiching a thin foil of polyethylene terephthalate from which the channel volume had been removed. Channel dimensions were 2.0 cm in breadth, 0.025 cm in thickness and 30 cm in length. The ensemble was sandwiched together using proper clamping systems, which may if necessary be removable clamping systems such as nuts, bolts or rivets (Fig. 1a).

The fractionation device should be prepared with the following instrumental set-up, as shown in Fig. 1b:

1. a peristaltic pump, used to impart the mobile phase into the system, was connected at the channel inlet by means of a T-valve;
2. the T-valve was connected to a PEEK inlet tube ($L=7 \text{ cm}$, $\text{i.d.}=0.750 \text{ mm}$, $\text{o.d.}=1/16''$) screwed at the beginning of the channel wall used to allow flow and sample injection.
3. at the fractionation device outlet, a UV/Vis detection system was connected to monitor the elution process, recording a signal at 600 nm;
4. a fraction collector was connected downstream of the detector outlet to collect eluted cells.
5. The overall system was placed in a laminar-flow hood to assess sterile conditions. (See Note 3).

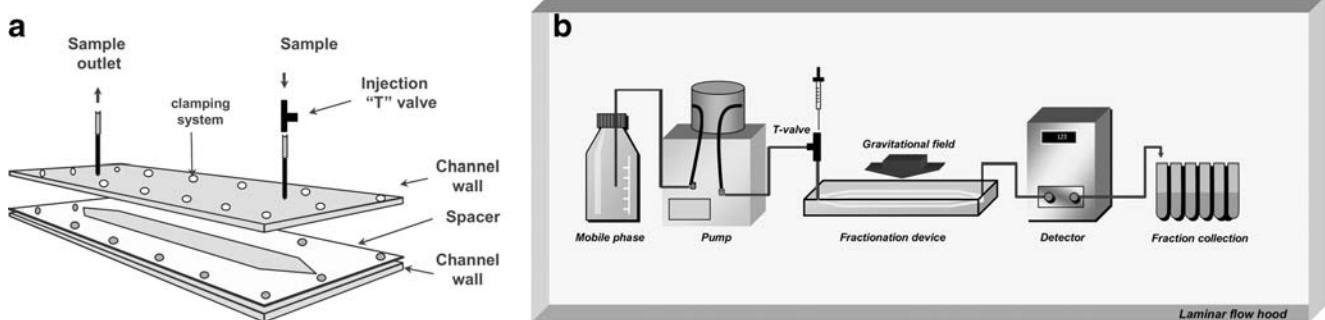


Fig. 1 a Fractionation device; b fractionation system instrumental set-up

Methods

Contamination from undesired cell populations frequently occurs in stem cell preparation. Phenotypically similar MSCs deriving from different sources showed differences in their biophysical characteristics. Within a heterogenous cell preparation, cells with a higher “stemness” are expected to show distinctive biophysical features.

The fractionation protocol allows tackling these issues.

Steps concerning sample preparation and fractionation protocol are here described.

Sample Preparation

MSCs are Isolated and Cultured from Different Sources

1. hMSCs are isolated from adult tissues like bone-marrow (BM), fetal membranes (FM), amniotic membrane (AM), dental pulp (DP) and adipose tissue (AT).

BM-MSCs are isolated by a Ficoll gradient [21].

All other MSC isolation procedures include mechanical and enzymatic digestion and exploit the MSC property of adhering to the culture flask [8,9].

2. MSCs are maintained in a culture medium composed of DMEM with 10% FBS, harvested and expanded by treatment with Trypsin-EDTA.
3. Culture passages and population doublings should be recorded.
4. hMSCs need to be tested for mesenchymal multi-differentiation potential (adipogenic, osteogenic, and chondrogenic) in accordance with established procedures [11]. (See Note 1)

Isolation and Culturing AECs from Amniotic Membrane

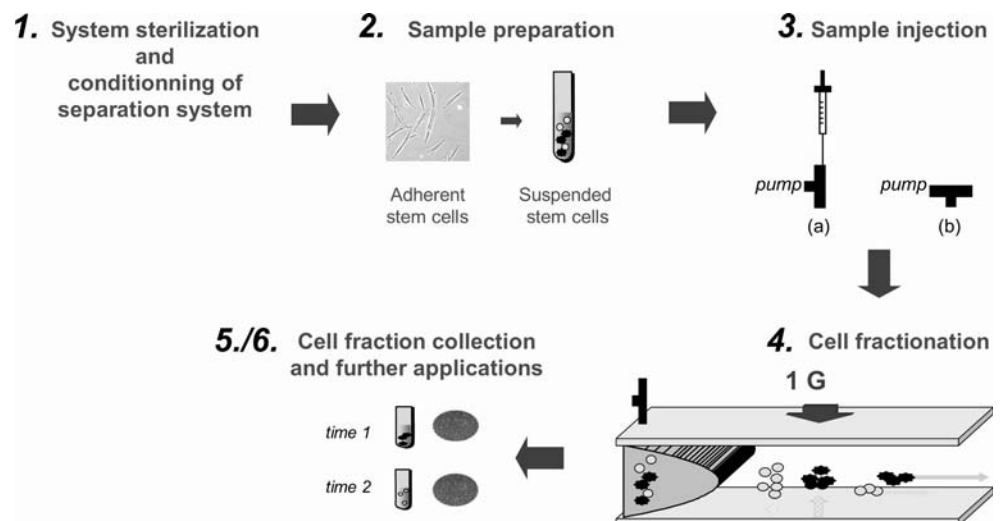
1. Amniotic membrane from term placentas is also processed for amniotic epithelial cell (hAEC) isolation, as previously described [8].
2. When epithelial cells are near confluence they are detached by enzymatic treatment with Trypsin-EDTA solution and then maintained in DMEM 10% FBS with 10 ng/ml EGF for expansion and *in vitro* assays.
3. Culture passages and population doublings are recorded.

Fractionation Protocol

The protocol (Fig. 2) consists of:

1. Sterilization of the fractionation system and conditioning to be performed at the beginning of each working day.
2. Fill the fractionation system with the sterilization solution for 1 h at 1 ml/min.
3. Fill the fractionation system with sterile water for 1 h at 2 ml/min to thoroughly wash the system and eliminate active chlorine traces.
4. Fill the fractionation system with sterile mobile phase for 30 min at 0.5 ml/min before sample injection for channel wall conditioning. (See Note 4 and 5)
5. The 100- μ L HPLC syringe to be used for sample loading was itself sterilized with the same hypochlorite solution, then washed twice with sterile water and finally with sterile mobile phase.
6. Preparation of a cell sample: cells were counted and resuspended in the mobile phase at a concentration of 3×10^5 cells/ml. Cells needed to be properly main-

Fig. 2 Fractionation protocol



tained in suspension to avoid cell aggregation. (See Note 2)

7. Injection of cell sample: a volume of 50 μL of the suspension was injected into the channel PEEK inlet tube ($L=7$ cm, i.d.=0.750 mm, o.d.=1/16") by means of an HPLC syringe. The flow was stopped for 3 seconds to allow the sample to enter the channel; then by means of a T-valve the inlet port was closed.
8. Elution of cell sample: after injection, the flow was immediately restarted and set at 0.46 ml/min. After a relatively short period of time from injection (about 30 min), cell elution was complete.
9. Cell fraction collection: when necessary, eluted cells were collected at the fractionation device outlet as selected fractions. (See Note 6)
10. Isolation of fractionated cells, and possibly further characterization/selection and/or *in vitro* expansion thereof.

Application

This protocol was used for: 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.

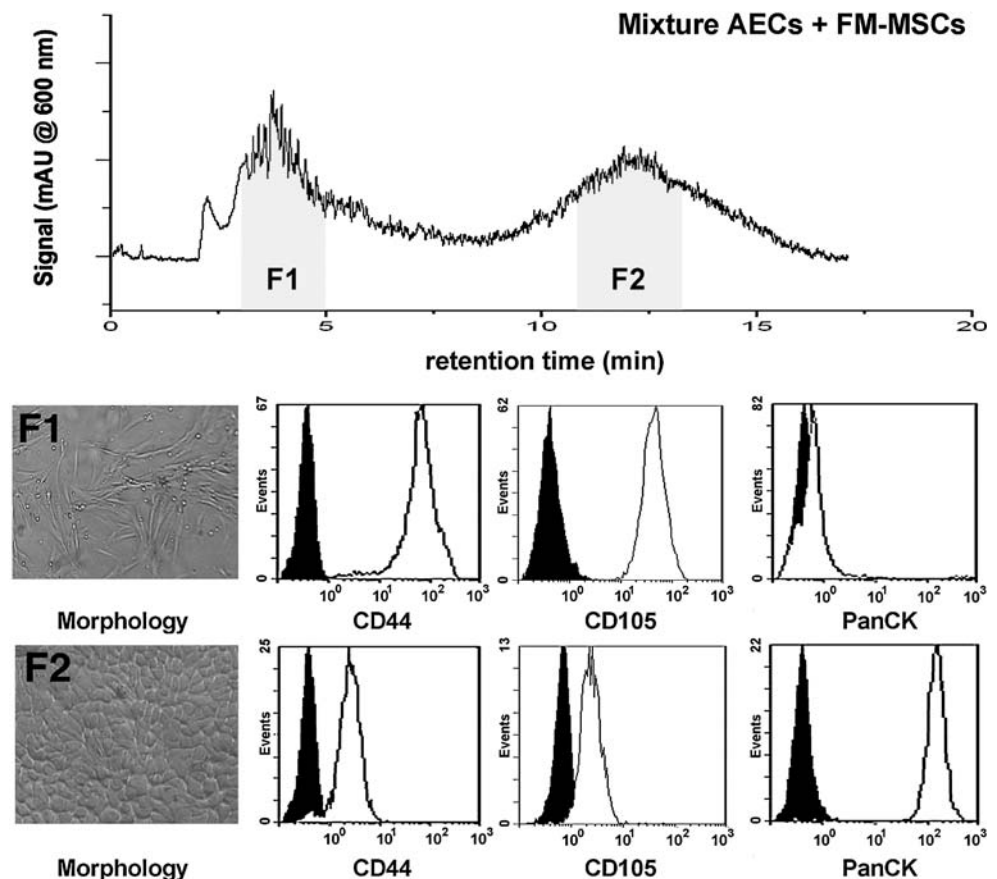
Purification of Fetal Membrane-Derived h-MSCs

1. The heterogeneous population of MSCs and AECs was 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 Fig. 3.
2. 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.
 - Cells collected in each fraction were pooled from three repeated runs.
3. F1 and F2 were subjected to **microscopic and flow cytometric characterization**.

Microscopic analysis of collected fractions:

F1 cells displayed the classic fibroblast-like morphology of hMSCs; F2 cells, conversely, showed cobblestone-shaped cells. Pictures of F1 and F2 cells are reported in Fig. 3.

Fig. 3 Fractogram obtained for a mixture of amniotic epithelial cells (AECs) and fetal membrane-derived hMSCs (FM-hMSCs) with F1 and F2 as collected fractions. Microscopic characterization: F1 collected cells displayed a fibroblast-like morphology; F2 cells displayed a cobblestone shape. Flow cytometric (CD44, CD105, PanCK) characterization: cells in F1 were found to be CD44^{high}, CD105^{high}, PanCK⁻; and cells in F2 were CD44^{low}, CD105^{low}, PanCK⁺. Reprinted with permission from [20] © J. Wiley & Sons



Flow cytometric characterization of collected fractions:

1. The immunophenotype of these cell populations was evaluated by flow cytometry using a panel of markers, following the manufacturer's instructions.
2. MSCs and AECs were characterized for expression of: CD29, CD34, CD44, CD45, CD73, CD90, CD105, CD166 and pan-Cytokeratin.
3. Background staining was evaluated by fluorochrome-matched isotype control antibodies.

4. Meanwhile Forward and Side Scatter data were acquired by flow cytometry.

As reported in Fig. 3, FC analysis of 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.

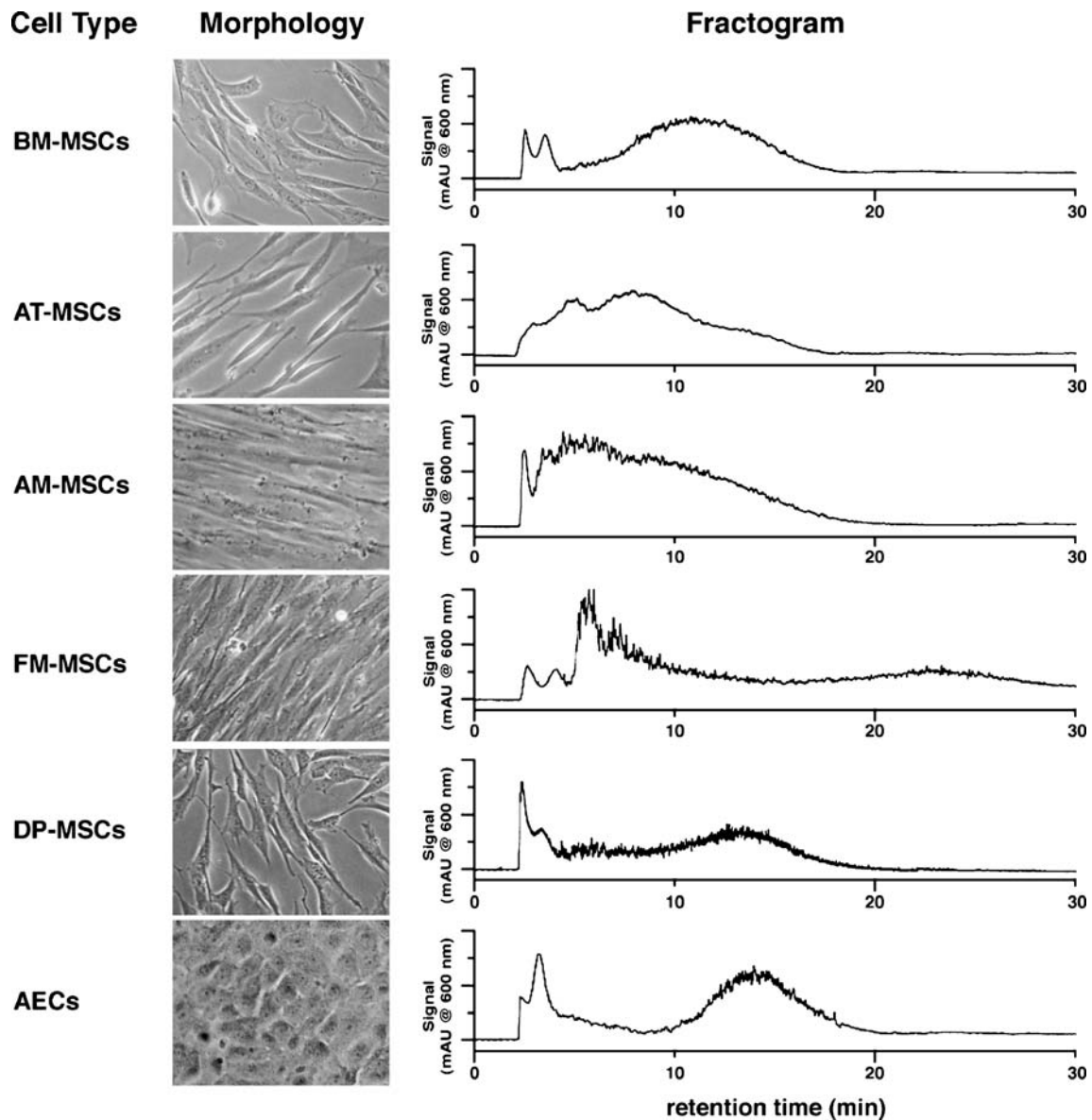


Fig. 4 Characteristic morphology and fractograms obtained for hMSCs isolated from different human sources. (BM-hMSCs: Bone Marrow hMSCs; AT-hMSCs: Adipose Tissue-derived hMSCs; AM-hMSCs: Amniotic Membrane hMSCs; FM-hMSCs: Fetal Membrane

hMSCs; DP-hMSCs: Dental Pulp hMSCs; AECs: amniotic epithelial cells). Differences observed in fractograms reflect differences in the biophysical features of stem cells originating from different sources

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 characterize phenotypically different MSCs. Results are shown in Fig. 4. These different profiles can be correlated with the different differentiation potential of such cells. In Fig. 4, the morphology of cell populations from different sources are also shown.

Sorting of hMSCs from a Single Source

- hMSCs obtained from adipose tissue were fractionated by the fractionation protocol giving a well-defined fractographic profile, as shown in Fig. 5.
- Four fractions (F1, F2, F3, F4) were collected:
 - 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.
- Cells from each fraction and from an unfractionated control (UC) were subjected to adipogenic differentiation: cells were seeded in 6-well plates at a density of 17×10^3 cells/cm².
- Cell induction proceeded for 3 weeks with induction medium changes every 3 days.
- The differentiation yield of the subpopulations was investigated by microscope after Red Oil staining. Results are reported in Fig. 5.

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.

Notes

Concerning the **sample preparation**, some cautions should be considered to optimize the fractionation protocol:

- MSCs retain multidifferentiation potential up to the 5th culture passage.
- Cell aggregation must be avoided by preparing a single cell suspension in polypropylene tubes and by continuously resuspending cells, at room temperature. These conditions guarantee high cell recovery and maintenance of their viability.

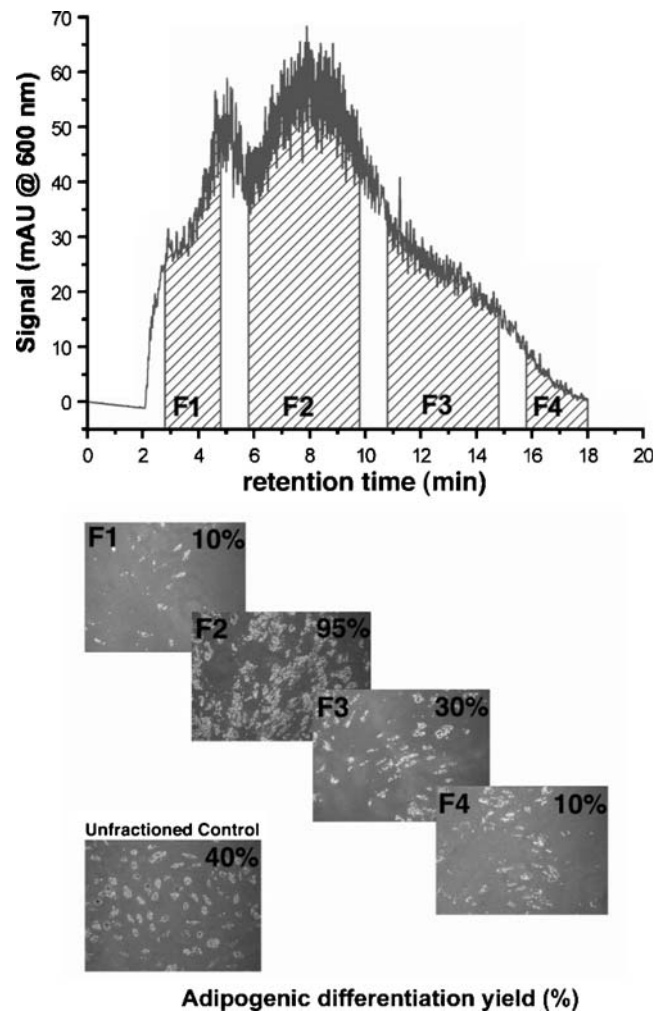


Fig. 5 Fractogram obtained for hMSCs isolated from human adipose tissue with F1, F2, F3, F4 as collected fractions. Microscope observation after Red Oil staining of F1-F4 after induction toward adipogenic differentiation. Sorted subpopulations displayed major differences in their adipogenic differentiation yield (%). Reprinted with permission from [20]. © J. Wiley & Sons

Concerning the **fractionation protocol**, the following critical points should be considered:

- the fractionation system has to be placed in a horizontal position to make the gravitational field act perpendicularly homogeneously to the carrier liquid flow.
- To set up the fractionation system properly, the conditioning procedure must be performed at the established time and flow rate. These procedures allow one to block any adhesion sites on the plastic channel walls and to eliminate any contamination traces on channels which could lead to contamination, recovery and decreased viability.
- Sterile mobile phase has to be used at room temperature and air bubbles inside the separation device have to be eliminated before fractionation of cell samples.
- Fractionation must be performed at room temperature; cells must not be kept on ice and incubated at 37°C 5%

CO₂ as soon as possible or appropriately prepared for characterization (e.g. fixation for flow cytometry).

References

- Wagner, W., Wein, F., Seckinger, A., Frankhauser, M., Wirkner, U., Krause, U., et al. (2005). Comparative characteristics of mesenchymal stem cells from human bone marrow, adipose tissue, and umbilical cord blood. *Experimental Hematology*, *33*, 1402–1416.
- Tong, J., Hoffman, R., Siena, S., Srour, E. F., Bregini, M., & Gianni, A. M. (1994). Characterization and quantification of primitive hematopoietic progenitor cells present in peripheral blood autograft. *Experimental Hematology*, *22*, 1016–1024.
- Lucas, A., Lepage, F., & Cardot, P. (2000). Cell separations. In M. E. Schimpf, K. Caldwell & J. C. Giddings (Eds.), *Field-flow fractionation hand-book, chapter 29* (pp. 471–486). New York: Wiley-Interscience.
- Reschiglian, P., Zattoni, A., Roda, B., Michellini, E., & Roda, A. (2005). Field-flow fractionation and biotechnology. *Trends in Biotechnology*, *23*, 475–83.
- Bernard, A., Paulet, B., Colin, V., & Cardot, P. (1995). Red Blood cell separations by gravitational field-flow fractionation: instrumentation & application. *Trends in Analytical Chemistry*, *14*, 266–273.
- Roda, B., Reschiglian, P., Zattoni, A., Tazzari, P. L., Buzzi, M., Ricci, F., et al. (2008). Human lymphocyte sorting by gravitational field-flow fractionation. *Analytical and Bioanalytical Chemistry*, *392*, 137–145.
- Roda, B., Reschiglian, P., Alviano, F., Lanzoni, G., Bagnara, G. P., Ricci, F., et al. (2009). Gravitational field-flow fractionation of human hemopoietic stem cells. *Journal of Chromatography A*, . doi:10.1016/j.chroma.2009.07.024.
- Parolini, O., Alviano, F., Bagnara, G. P., Bilic, G., Bühring, H. J., Evangelista, M., et al. (2008). Concise review: isolation and characterization of cells from human term placenta: outcome of the first international Workshop on Placenta Derived Stem Cells. *Stem Cells*, *26*, 300–311.
- Marchionni, C., Bonsi, L., Alviano, F., Lanzoni, L., Di Tullio, A., Costa, R. (2009). Angiogenic potential of human dental pulp stromal (stem) cells. *International Journal of Immunopathology and Pharmacology*, in press.
- Pittenger, M. F., Mackay, A. M., Beck, S. C., Jaiswal, R. K., Douglas, R., Mosca, J. D., et al. (1999). Multilineage potential of adult human mesenchymal stem cells. *Science*, *284*, 143–147.
- Alviano, F., Fossati, V., Marchionni, C., Arpinati, M., Bonsi, L., Franchina, M., et al. (2007). Term Amniotic membrane is a high throughput source for multipotent Mesenchymal Stem Cells with the ability to differentiate into endothelial cells in vitro. *BMC Developmental Biology*, *21*, 7–11.
- Pasquinelli, G., Tazzari, P. L., Vaselli, C., Foroni, L., Buzzi, M., Storci, G., et al. (2007). Thoracic aortas from multiorgan donors are suitable for obtaining resident angiogenic mesenchymal stromal cells. *Stem Cells*, *2007*(25), 1627–1634.
- Zuk, P. A., Zhu, M., Ashjian, P., De Ugarte, D. A., Huang, J. I., Mizuno, H., et al. (2002). Human adipose tissue is a source of multipotent stem cells. *Molecular Biology of the Cell*, *13*, 4279–4295.
- Uccelli, A., Pistoia, V., & Moretta, L. (2007). Mesenchymal stem cells: a new strategy for immunosuppression? *Trends in Immunology*, *28*, 219–226.
- Jiang, Y., Jahagirdar, B. N., Reinhardt, R. L., Schwartz, R. E., Keene, C. D., Ortiz-Gonzalez, X. R., et al. (2002). Pluripotency of mesenchymal stem cells derived from adult marrow. *Nature*, *418*, 41–49.
- Baksh, D., Song, L., & Tuan, R. S. (2004). Adult mesenchymal stem cells: characterization, differentiation, and application in cell and gene therapy. *Journal of Cellular and Molecular Medicine*, *8*, 301–316.
- Musina, R. A., Bekchanova, E. S., Belyavskii, A. V., & Sukhikh, G. T. (2006). Differentiation potential of mesenchymal stem cells of different origin. *Bulletin of Experimental Biology and Medicine*, *141*, 147–151.
- Pasquinelli, G., Tazzari, P., Ricci, F., Vaselli, C., Buzzi, M., Conte, R., et al. (2007). Ultrastructural characteristics of human mesenchymal stromal (stem) cells derived from bone marrow and term placenta. *Ultrastructural Pathology*, *31*, 23–31.
- Reschiglian, P., Roda, B., Zattoni, A., & Bagnara G. P. (2007). Method and device to fractionate stem cell. Patent pending PCT/EP2007/054226.
- Roda, B., Reschiglian, P., Zattoni, A., Alviano, F., Lanzoni, G., Costa, R., et al. (2009). A tag-less method of sorting stem cells from clinical specimens and separating mesenchymal from epithelial progenitor cells. *Cytometry Part B (Clinical Cytometry)*, *76B*, 285–290.
- Biddison, W. E. (2001). *Curr Protoc Cell Biol*, Capter 2, Unit 2.2.