ORIGINAL PAPER

RJME
Romanian Journal of Morphology & Embryology
http://www.rjme.ro/

Morphological and functional characterization of femoral head drilling-derived mesenchymal stem cells

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Abstract

Adult mesenchymal stem cells (MSCs) were primary identified as bone marrow-derived cells, fibroblast-like morphology, and adherent to plastic surfaces of *in vitro* culture plate. Their identification criteria evolved in time to a well-established panel of markers (expression of CD73, CD90, and CD105) and functional characteristics (adipogenic, osteogenic, and chondrogenic trilineage differentiation ability), which can be applied to adult mesenchymal stem cells obtained from other tissue sources. We tried to assess the potential stemness of femoral head drilling-derived cells as a new source of mesenchymal stem cells (FH-MSCs). For this purpose, we used the morphological and ultrastructural characteristics defined by scanning and transmission electron microscopy and spindle-shape cellular body, fibroblast-like, with few thick elongations (lamellipodia) and numerous fine, thin cytoplasmic projections (filopodia) that extend beyond the edge of lamellipodia. Immunophenotypical analysis was performed by flow cytometry and immunocytochemical methods and we showed that FH-MSCs share the characteristic markers of MSCs, expressing CD73, CD90, CD105, and being positive for vimentin, and c-kit (CD117). Proliferation rate of these cells was moderate, as revealed by Ki67 immunostaining. Regarding the functional characteristics of FH-MSCs, after appropriate time of induction in specific culture media, the cells were able to prove their trilineage potential and differentiated towards adipocytic, osteogenic, and chondrogenic lineage, as revealed by immunofluorescent staining. We may conclude that femoral head drilling-derived cells can be used as a novel source of stem cells, and employed in diverse clinical settings.

Keywords: femoral head drilling-derived mesenchymal stem cells (FH-MSCs), ultrastructural characterization, immunophenotypical markers, trilineage potential.

Introduction

More than 40 years ago, Friedenstein *et al.* [1] reported existence of bone marrow-isolated fibroblast-like cells, which were adherent to plastic surfaces of culture plates, *in vitro*. They described a cellular population consisting of multipotent precursor stromal cells, spindle-shape like, with clonogenic ability, which were further named colony-forming unit fibroblast (CFU-F). However, these cells were named mesenchymal stem cells (MSCs) much later, and were further characterized by their capacity to differentiate into adipocytes, chondrocytes, osteoblasts, and myoblasts, both *in vitro* and *in vivo*. Moreover, their ability to differentiate into cardiomyocytes, neurons, and astrocytes was explored and confirmed, *in vitro* and *in vivo* [2–5]. Nevertheless, there are still many unanswered questions regarding MSCs identity, including localization, origin, and their multipotent ability.

Successful isolation of MSCs was performed from multiple tissue sources, such as adipose tissue [6], liver [7], skeletal muscle [8], amniotic fluid and placenta [9], umbilical cord blood [10], and dental pulp [11], but bone marrow remains the main source of MSCs for pre-clinical and clinical studies.

The true identity of MSCs was questioned in laboratories working with stem cells and using different isolation protocols and diverse *in vitro* culture methods. These variables are responsible of the phenotype and function of the resulting cellular populations. It is not clear if these conditions promote selectively expansion of different MSCs populations, or if they induce acquirement of different phenotypes by similar cellular populations.

It is estimated that MSCs represent only 0.001% and 0.01% of total nucleated cells from bone marrow aspirates, counting for less than 10 times the population of hematopoietic stem cells [12]. Despite their reduced number, the general interest for MSCs remains high, due to their properties: can be easily isolated in small amounts from bone marrow aspirate, and can be expanded in culture, reaching 40 population-doubling times in 8–10 weeks.

MSCs isolated from different sources were studied, each type being endowed with diverse proliferation and differentiation capacities. The progress regarding MSCs studies was impaired by the fact that researchers could not find a specific membrane/cytoplasmic marker for identification of these cells. Reference papers published by *International Society of Cellular Therapies* tried to clarify terminology related to MSCs, by addition of cellular source, and also to establish minimal criteria, which are used for defining human adult mesenchymal stem cells (MSCs) [13].

MSCs can be found in specialized niches of different

tissues, and it was demonstrated that bone marrow and spleen represent engraftment sites for these cells [14, 15]. Small amount of MSCs are found constantly within the circulation in peripheral blood in basal conditions, while under hypoxic conditions their count increases very much [16]. However, numerous studies reported controversial results, such as even failure to isolate MSCs from peripheral blood [17].

MSCs utilization in clinical applications were intensively studied due to presumption that these cells have homing capacities towards inflammatory lesions. Chapel *et al.* [18] demonstrated in a multiple organ deficiency model performed on primates (macaque) that GFP-labeled MSCs migrate towards tissues directly proportional with lesions extent. Homing is the process by which cells are migrating and engraft in tissues where will perform their functional and protective effects. MSCs homing capacity leads to avoidance of possible complications associated with local or intra-muscular injection, such as bone formation, in autologous and allogeneic transplantation procedures, required for regenerative medicine [19].

Present study aims to characterize a new population of cells – femoral head drilling-derived – and to investigate whether these cells have the entire panel of mesenchymal stem cells features. Being a cellular source that yields increased number of cells they could be used in clinical studies based on their immunomodulatory function and tissue regeneration potential.

Materials and Methods

Isolation of femoral head drilling-derived mesenchymal stem cells (FH-MSCs) (direct adherence method)

This method is different from bone marrow-derived MSCs isolation because the femoral head is drilled and the content is aspired into a sterile syringe. After harvesting, the sample is successively passed through cell strainers of 100 and 70 μL, for removal of bone fragments. After these additional steps, the sample is processed similar to bone marrow in order to obtain stem cells. Shortly, cellular suspension is 1:1 diluted with PBS (Phosphate Buffered Saline, Sigma-Aldrich Company, St. Louis, Missouri, USA) and transferred into sterile 50 mL tubes, followed by a centrifugation step for 10 minutes at 1800 rpm, room temperature. Cell pellet is mechanically homogenized and resuspended in cell culture medium containing MEM Alpha Medium (Gibco, NY, USA), 10% FCS (Newborn Calf Serum, Sigma-Aldrich Company), and 1% Penicillin/Streptomycin antibiotic solution (Sigma-Aldrich Company). Cell plating density should not exceed 1×10^5 cells/cm^2. Medium replacement was performed every three days, and cells are grown in humid atmosphere containing 5% CO_2 at 37^0C, until reaching 80–90% confluence.

Morphological and ultrastructural characterization of femoral head drilling-derived MSCs

Scanning electron microscopy (SEM)

Scanning electron microscopy was performed for identification of morphological characteristics of cells obtained from femoral head drilling. Cells were cultured at 7000 cells/cm^2 in 24-well format cell culture inserts (BD Labware Europe, Le Pont De Claix, France) and left to adhere and expand for 48 hours until reaching the appropriate confluence (80–90%). Cells were pre-fixed for one hour with 2.5% buffered Glutaraldehyde (in PBS, Sigma-Aldrich Company). For better image quality, cells fixed on the membranes were sputter-coated with platinum–palladium and examined with a FEI Quanta 3D FEG electron microscope (FEI Company, Eindhoven, Nederland) generating digital electron micrographs.

Transmission electron microscopy (TEM)

FH-MSCs ultrastructural morphology was assessed by TEM, 48 hours after plating in appropriate culture flasks. Cells were pre-fixed for one hour with Glutaraldehyde (2.5% in PBS), rinsed three times in PBS, post-fixed for one hour in Osmium Acid (2% in PBS), dehydrated and infiltrated with Epon resin. Sections of about 100 nm, obtained on a diamond knife (Diatome) with Leica UC6 ultramicrotome (Leica Microsystems Inc., LKB-II, Germany) were post-stained with lead citrate and uranyl acetate. The grids were examined with a FEI Tecnai 12 transmission electron microscope (FEI Company).

Analysis of immunophenotypical profile of FH-MSCs

Flow cytometric methods

FH-MSCs in culture reaching 80–90% confluence were detached, washed and stained with mouse anti-human conjugated antibodies following the manufacturer instructions (BD Biosciences, Heidelberg, Germany). Conjugated antibodies utilized included PE-conjugated CD14 (BD Pharmingen™), CD117 (BD Pharmingen™), CD29, CXCR4 (R&D Systems, Minneapolis, MN, USA), as well as FITC-conjugated CD34, CD44, CD45, CD73, CD90, CD106, and HLA-DR (BD Pharmingen™), acquisition was performed on FACSCalibur flow cytometer (Becton-Dickinson), while data were analyzed using FlowJo v.7.6 dedicated software.

Immunohistochemical/immunofluorescence analysis

Immunohistochemistry was performed for FH-MSCs obtained from femoral head drilling. Cells prepared for these analyses were fixed with formalin and investigated for expression of interest proteins, employing the following antibodies: monoclonal mouse anti-swine vimentin (clone V9), monoclonal mouse anti-human endoglin, CD105 (clone SN6h), monoclonal mouse anti-human Ki-67 antigen (clone MIB-1), and polyclonal rabbit anti-human CD117 (c-kit), respectively. All primary antibodies were provided by DakoCytomation (Denmark) and tested for human specificity and cross-reactivity. Staining protocol continued with secondary biotinylated antibody binding, substrate addition, and Hematoxylin counterstaining of the nuclei (Dako EnVision™ Systems-HRP) following the manufacturer procedures.

Femoral head drilling-derived MSCs were employed in differentiation experiments towards adipocytes, chondrocytes and osteoblasts, while for identification of their trilineage differentiation potential we used as primary

antibody anti-mFABP4, anti-hAggrecan, and anti-hOsteocalcin, respectively from Human Mesenchymal Stem Cell Functional Identification Kit (R&D Systems). The visualization system was obtained by coupling the primary antibody with specific fluorochrome-conjugated secondary antibody (AlexaFluor 546/488/594, respectively).

Differentiation potential of femoral head drilling-derived MSCs

The trilineage potential of FH-MSCs to differentiate into adipogenic, osteogenic and chondrogenic lineages was assessed at different passage levels, starting with passage 2. Non-hematopoietic stem cell medium for generation of osteoblasts, chondrocytes and adipocytes (Miltenyi Biotec) was used, supplemented with 1% Penicillin/Streptomycin mixture. Adipogenic differentiation was revealed 21 days after medium induction, osteogenic potential after 10 days, while chondrogenic differentiation takes around 24 days of growth in specific medium.

Results

Morphological and ultrastructural characteristics of femoral head drilling-derived MSCs

Analysis using scanning electron microscopy (SEM) showed that femoral head drilling-derived MSCs have a spindle-shape cellular body, fibroblast-like, with few thick elongation (lamellipodia) and numerous fine, thin cytoplasmic projections (filopodia) that extend beyond the edge of lamellipodia. These types of cellular elongations are specific for migrating cells, being involved in sensing the chemotropic cues and directing cellular locomotion (Figure 1).

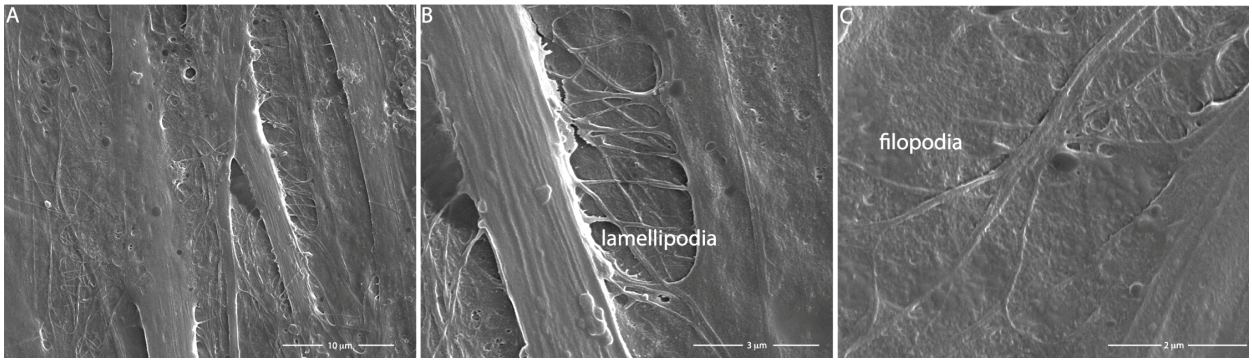

Figure 1 – ***Scanning electron microscopy (SEM) of femoral head drilling-derived MSCs in culture: (A) General aspect of MSCs in vitro cell culture showing shape and adherence of these cells; (B) Two thick elongations (lamellipodia) connected through thinner projections from each cell; (C) Details of thin cellular projections forming focal adhesions with the substratum, linking it to the cell surface (filopodia).***

In transmission electron microscopy (TEM), FH-MSCs present typical ultrastructure, with large nucleus, containing high amount of euchromatin. Nuclei are of irregular shape, with discrete indentations and easily visible nucleoli (Figure 2B). Within the cytoplasm, there are dilated cisternae of rough endoplasmic reticulum, glycogen aggregates, numerous mitochondria and clear content vacuoles (Figure 2, D and E). Cellular cytoskeleton is formed of intermediate filaments, which are also visible in TEM and cellular products secreted by extracellular matrix (Figure 2C). The average size of FH-MSCs is 15–20 µm (Figure 2A) and external cell surface is dominated by cytoplasmic elongations, which have a tendency of grouping.

Immunophenotypical characteristics of femoral head drilling-derived MSCs

Femoral head drilling-derived MSCs are positive (>98%) for specific markers – CD73, CD90, CD105 – and negative (<2%) for membrane markers whose expression is described in literature as being very low – CD11b, CD34, CD45. However, CD90+ cellular population is divided in two subpopulations, suggesting that some FH-MSCs are more mature than others, CD90 being a marker of cellular immaturity. Figure 3 depicts a representative example of flow cytometric analysis for FH-MSCs phenotype. FH-MSCs were also positive in fewer amounts (4.56%) for membrane expression of CD117 (data not shown). Even though MSCs are generally defined by lack of HLA-DR, for FH-MSCs we detected low level of HLA-DR expression, for all cellular passages investigated (data not shown). This confirms activated status of these cells. We were not able to detect E-cadherin and cytokeratin expression, either by flow cytometric or immunocytochemical methods.

Immunocytochemical analyses revealed presence of other cytoplasmic phenotypical markers, such as endoglin (CD105), vimentin, and stem cell factor receptor (c-kit, CD117). Vimentin and CD105 are also described as mesenchymal stem cell markers, and the femoral head drilling-derived cells have increased expression of these markers, more than 98% of the cells, counted on five different microscopic fields (Figure 4, A and C). On the other hand, we were able to identify presence of CD117, c-kit in large proportion of cells (more than 50%), which completes the phenotypical profile of these cells (Figure 4C). CD117 was not detected by flow cytometric methods, probably due to internalization of the antigen during the procedural steps. Ki67 (proliferation factor) was moderately expressed at nuclear level in FH-MSCs (30%), suggesting that these cells do not have an increased proliferative ability (Figure 4B).

Differentiation studies of femoral head drilling-derived MSCs

We performed FH-derived MSCs differentiation towards three mesodermal cellular lines – adipogenic, osteogenic, and chondrogenic. As the BM-derived MSCs,

our cells isolated from femoral head drilling are endowed with *in vitro* trilineage plastic capacity, thus confirming the characteristics of multipotent stem cells.

Differentiation degree towards each specialized lineage is variable, dependent on cellular type they are induced to differentiate into. Osteoblastic differentiation is revealed in 90% of the FH-MSCs by staining with Osteocalcin intracellular marker (Figure 5A), while the chondrogenic differentiation is performed only by approximately 50% of the cells (Figure 5B). However, we must specify that the usual chondrogenic *in vitro* differentiation is performed on pelleted cells, while we induced this differentiation type on monolayer cell culture, which could result in a lower differentiation rate. Adipogenic differentiation was revealed in 90% of the FH-MSCs by FABP4 (fatty acid binding protein 4) cytoplasmic staining (Figure 5C).

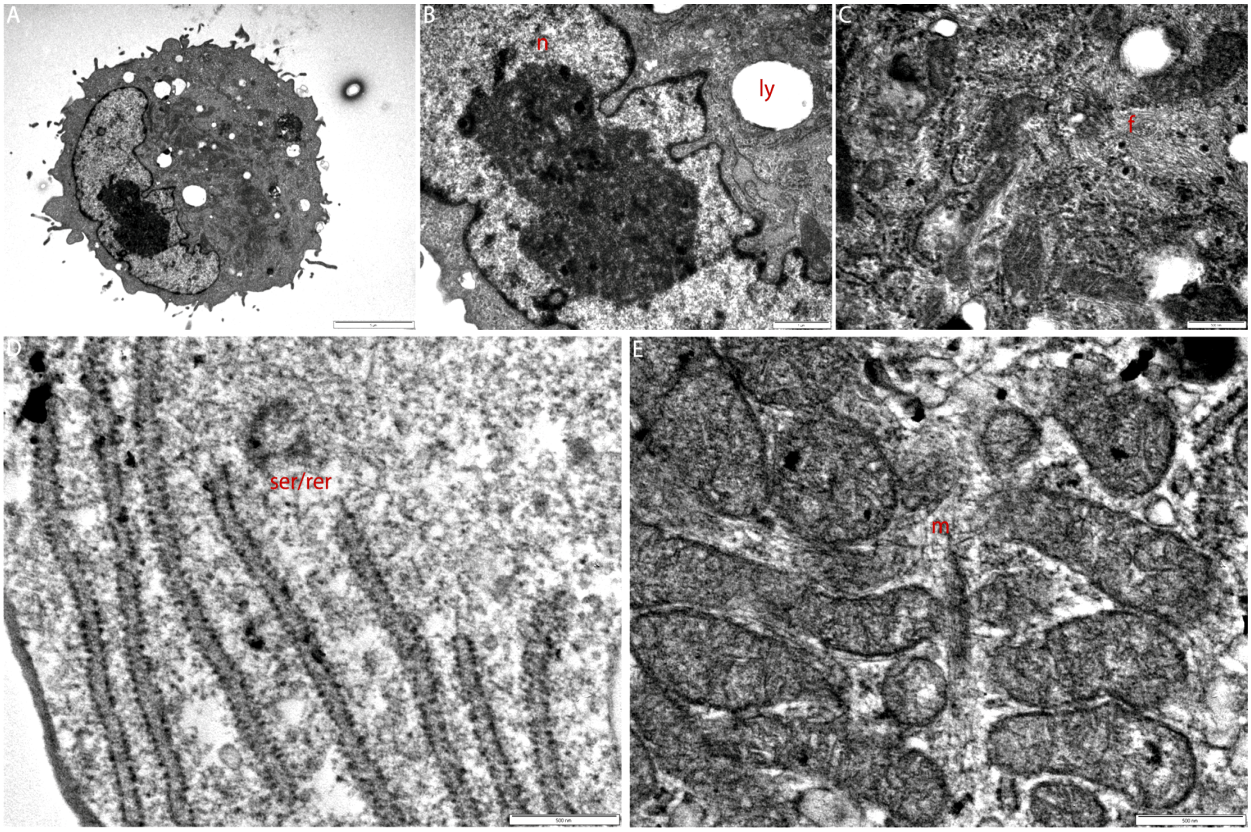

Figure 2 – ***Transmission electron microscopy (TEM) of femoral head drilling-derived MSCs in culture: (A) General aspect of MSCs, showing numerous elongations and medium-size of these cells; (B) Large nucleus with discrete indentations (n), visible nucleoli and few lysosomal vesicles (ly); (C) Abundant intermediate filaments (f), with well-established architecture; (D) Intracytoplasmic smooth and rough cisternae of endoplasmic reticulum (ser/rer); (E) Numerous mitochondria (m).***

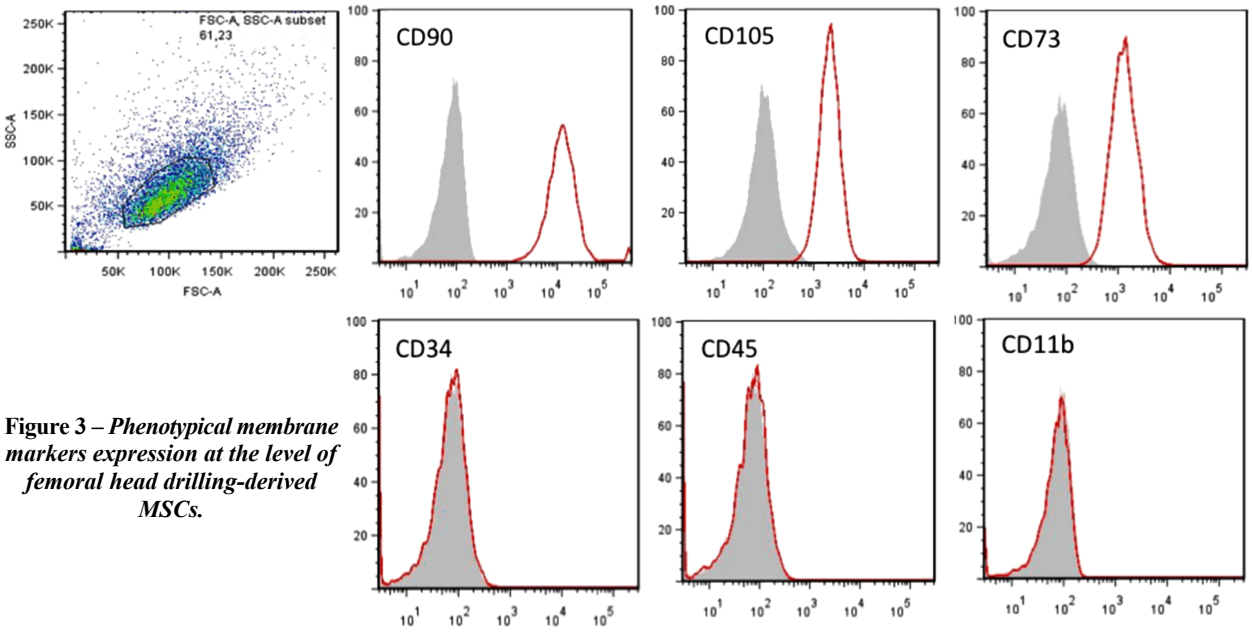

Figure 3 – ***Phenotypical membrane markers expression at the level of femoral head drilling-derived MSCs.***

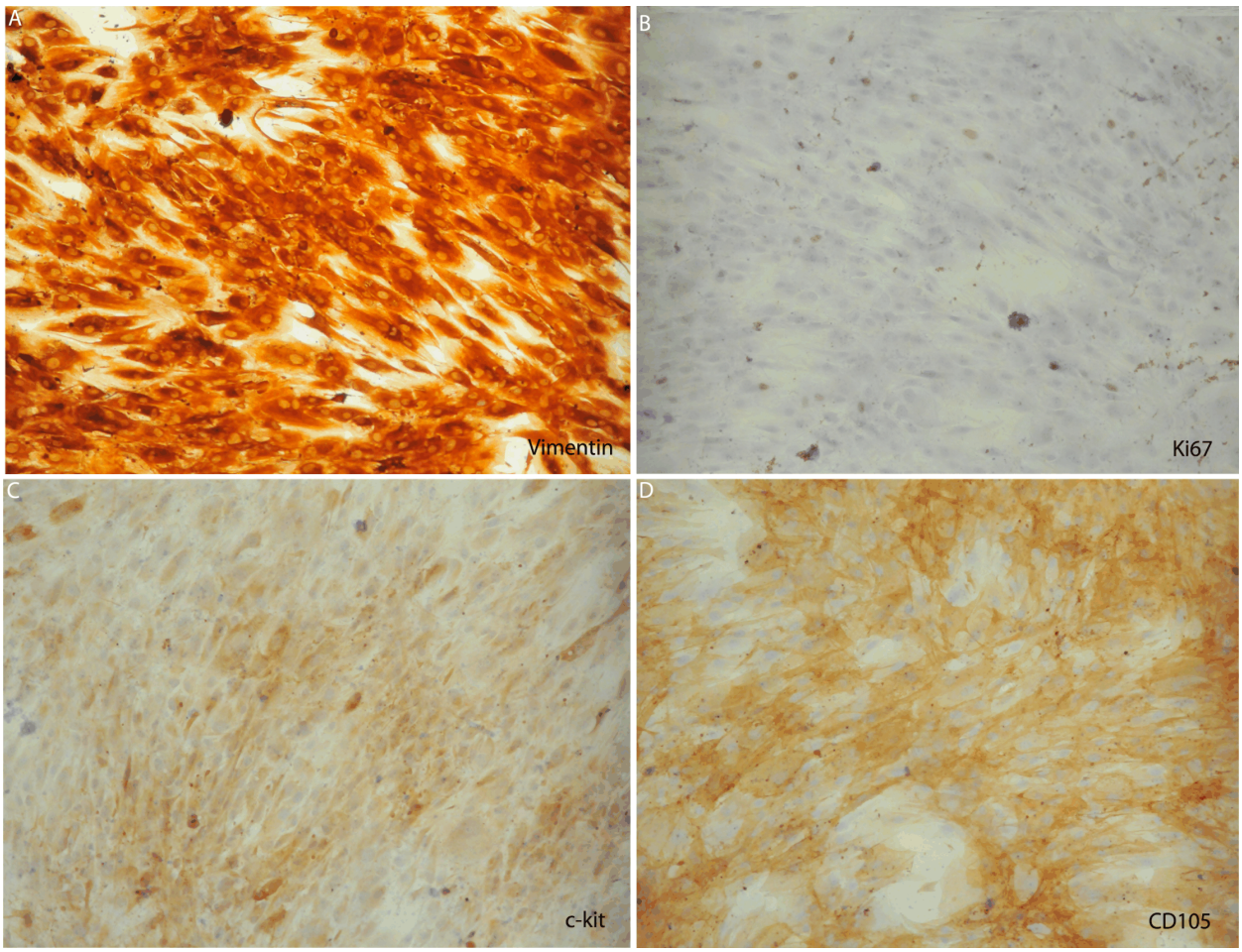

Figure 4 – ***Expression of phenotypical markers of MSCs in femoral head drilling-derived MSCs cultivated in vitro. FH-MSCs express vimentin, CD117/c-kit, CD105, and retain a reduced proliferation rate suggested by low expression of Ki67 nuclear marker.***

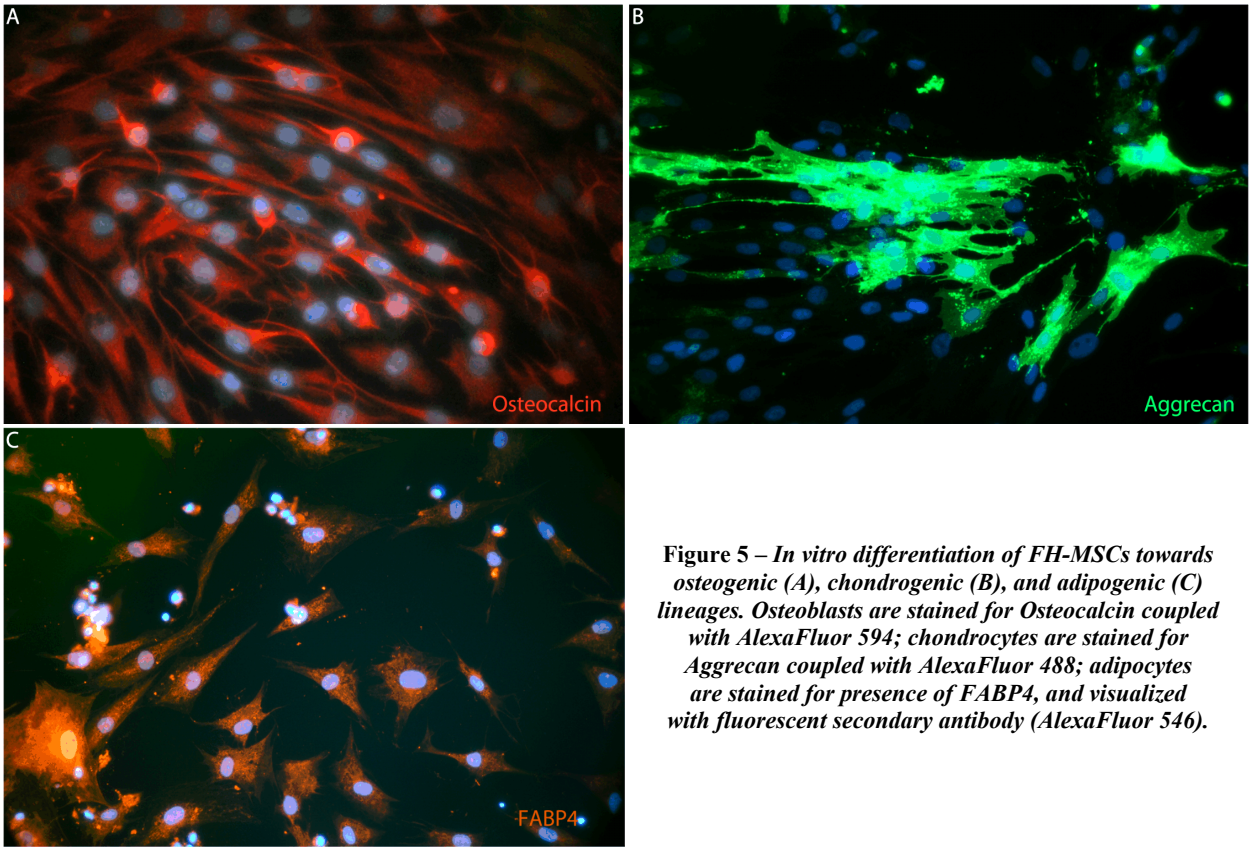

Figure 5 – ***In vitro differentiation of FH-MSCs towards osteogenic (A), chondrogenic (B), and adipogenic (C) lineages. Osteoblasts are stained for Osteocalcin coupled with AlexaFluor 594; chondrocytes are stained for Aggrecan coupled with AlexaFluor 488; adipocytes are stained for presence of FABP4, and visualized with fluorescent secondary antibody (AlexaFluor 546).***

Discussion

MSCs morphology is well known in optic microscopy. They are cells adherent to plastic surfaces, with fibroblast-like shape, have numerous elongations, and form spindles when reaching confluence [20]. However, ultrastructural details of MSCs are not very well known [21], compared to differentiation capacity and phenotypic characteristics of these cells. These characteristics depicted by our study for FH-derived MSCs could represent the bases of in situ identification of cellular niches in which these cells are residing and provide functional support for maturation of cellular precursors of adult tissues. SEM was very rarely used for studying ultrastructural details of MSCs, and only for osteogenic differentiation induced by different biostructures [22, 23].

Using TEM, FH-MSCs revealed few ultrastructural morphological characteristics, which distinguish them from other cellular types, but showed similarities with BM-derived MSCs: dilated cisternae of rough endoplasmic reticulum, vacuoles, and polypodia. The first characteristic is correlated with mesenchymal nature of stem cells, thus indicating that even FH-MSCs are cells involved in intense protein synthesis, required for cellular growth and differentiation. Important electrono-clear vacuoles representation within the cytoplasm can suggest intense endocytic activity, but this may not be the only explanation because lysosomes are not present in sufficient amount. It is possible that these vacuoles are folding or invaginations of cellular membrane, which can suggest an effect of cellular memory related to the original microenvironment in which these cells developed; in this case, these features can be useful in primary assessment of FH-MSCs morphology within their specific niche.

Discrepancy between flow cytometric assessment and immunocytochemical staining for CD117/c-kit can be explained by the fact that flow cytometric staining did not use a cellular permeabilization step, so that detection of this marker was revealed only at membrane level. However, CD117 is a predominantly intracellular molecule, being a transmembrane tyrosin-kinase receptor, binding stem cell factor (SCF). CD117/c-kit is expressed in numerous cellular types, being involved in development of stem cell lineages, such as germinal cells, neural crest-derived melanocytes, and hematopoietic precursor cells.

Vimentin is frequently used as marker of epithelial-mesenchymal transition, which happens during embryogenesis or metastasis process, but the implications of this type III filament are not completely known. It is speculated that this molecule is involved in protein transport between nucleus and cytoplasmic membrane. Vimentin is well represented in BM-derived MSCs, but also in our cellular population of FH-derived MSCs.

According to *International Society of Cellular Therapies* [13], the following are the minimum criteria to identify mesenchymal stem cells: positive membrane expression (over 98% of cells) for CD105, CD73, CD90 markers; lack of membrane expression (less than 2%) for CD34, CD45, CD11a, CD19, and HLA-DR markers; adherence to plastic surfaces of culture plate, in standard culture conditions; capacity to differentiate into adipocytes, osteoblasts, and chondrocytes, under specific *in vitro* stimuli. One of the key characteristics of MSCs is their plastic capacity, meaning *in vitro* potential to differentiate towards other cellular lineages, under appropriate cell culture conditions. Generally, the resulting differentiated cellular population is heterogeneous and do not resemble the target tissue from the biochemical and biomechanical perspective. Similar to BM-derived MSCs, the cells obtained from femoral head drilling exhibited the same plastic capacity.

Chondrogenic *in vitro* differentiation of MSCs is usually obtained by three-dimensional culture of cells, in pellet, in presence of growth and differentiation factors from TGFβ family [24, 25]. Nevertheless, differentiation of monolayer cell culture of FH-MSCs performed in our study resulted in similar processes. MSCs differentiation into cartilage is characterized by upregulation of specific genes, such as collagen II and IX, aggrecan, collagen biosynthesis and proteoglycans. Chondrogenesis involves many phases during embryogenesis: recruitment, migration, proliferation and *in vivo* condensation of MSCs [26]; these events are orchestrated by a series of growth factors and factors initiating (TGFβ, IGF, FGF) or suppressing signaling pathways, such as Hedgehog, Wnt, Notch, etc. [27]. Morphogenetic signals sent by chondrocytes are also involved in regulation of MSCs osteogenic differentiation [28, 29].

Similar to chondrogenesis, osteogenesis represents a set of well-orchestrated cellular processes, during which the cells are under direct supervision of soluble factors, such as cytokines and molecules from extracellular matrix [30]. We performed *in vitro* differentiation of FH-MSCs by addition in culture media of beta-glycerolphosphate, ascorbic acid, and other substances included in non-hematopoietic stem cell medium for generation of osteoblasts (Miltenyi Biotec). For monolayer cultured FH-MSCs, in high amount of Fetal Calf Serum, expression of Osteocalcin was increased, while the literature includes other changes of differentiated MSCs, such as upregulation of expression and activity for alkaline phosphatase, and increased deposits of mineralized matrix [4].

Regarding the adipogenesis, FH-MSCs differentiated in high proportion into adipocytes, similar to other MSCs from different tissue sources. We investigated the presence of FABP4 (fatty acid binding protein 4), but some other markers can be used for similar purposes, such as PPARγ (peroxisome proliferator activated receptor gamma) [31], lipoprotein lipase or secretion of adiponectin and leptin [32].

For FH-MSCs, we employed the differentiation media based on their composition, after analysis and synthesis from numerous protocols used for chondrogenic [25, 33], osteogenic [34, 35], and adipogenic differentiation [31, 36].

Numerous studies also demonstrated *in vitro* trans-differentiation of MSCs towards cells from ectodermal or endodermal layer, such as neuronal lineage [37], cardiomyocytes, type I pneumocytes, and epithelial cells [38–41]. However, the majority of *in vivo* studies showed low level of engraftment and MSCs transdifferentiation. Considering the difficulty of interpreting these experimental results, transdifferentiation is not considered as an option for MSCs clinical use, so that we did not perform any transdifferentiation experiments on FH-MSCs.

Conclusions

We identified a possible new source of mesenchymal stem cells, femoral head drilling-derived (FH-MSCs). We characterized these cells from morphological and functional perspective and concluded that they fulfill the minimum criteria, which are used for defining human adult mesenchymal stem cells (MSCs). Considering their *in vitro* potential, we suggest that these cells can be used in tissue regeneration clinical application, especially for osteochondral regeneration, both in autologous and allogeneic transplantation.

Acknowledgments

This work was supported by UEFISCDI, PNII–Idei, Project No. 318/2011.

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Received: March 12, 2014

Accepted: December 17, 2014