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Ultrastructural features of human adipose-derived multipotent mesenchymal stromal cells

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Abstract

Multipotent mesenchymal stromal cells (MMSCs) are plastic-adherent cells with a well-established phenotype. Equine, but not human, adipose MMSCs have been characterized ultrastructurally. The purpose of our study was to evaluate ultrastructurally the adipose-derived human MMSCs. Cell cultures were prepared from human lipoaspirate. The flow cytometry evaluation of surface markers of cultured cells confirmed the expected profile of MMSCs, that were positive for CD73, CD90 and CD105, and negative for CD34 and CD45. We examined these human adipose-derived MMSCs in transmission electron microscopy (TEM) by Epon en-face embedding the fixed MMSCs. The main ultrastructural features of MMSCs were the extremely rich content of endosomal/vesicular elements, long mitochondria, dilated RER (rough endoplasmic reticulum) cisternae, and abundant intermediate filaments and microtubules. We found two types of MMSCS prolongations: (a) thick processes, with opposite, vesicular and filaments-rich, sides and (b) slender processes (pseudopodes and filopodes), with occasional proximal dilated segments housing mitochondria, vesicles and secretory granules. These TEM features of MMSCs characterized an *in vitro* cell population and could use to distinguish between different cell types in culture.

Keywords: mesenchymal stem cells, flow cytometry, adipose-derived mesenchymal cells, transmission electron microscopy.

Introduction

Mesenchymal stem cells (MSC), which are the ideal cell model for cell therapies development [1], have an inconsistent nomenclature [2].

The *Mesenchymal and Tissue Stem Cell Committee* of the *International Society for Cellular Therapy* (ISCT) recommended a clarification of the nomenclature for mesenchymal stem cells (MSC) [3]. They was proposed that the plastic-adherent cells currently described as mesenchymal stem cells be coined multipotent mesenchymal stromal cells (MMSCs), and the term mesenchymal stem cell should describe a subset of these (or other) cells that prove stem cell activity by clearly stated criteria. For both cell populations, the acronym MSC may be used; thus, it is imperative that investigators unequivocally define the acronym in presentations of their work. The plastic adherence of these cells limits however their assignment only to *in vitro* media.

Also, ISCT Committee proposed minimal criteria to define human MMSCs. First, these cells must be plastic-adherent when maintained in standard culture conditions. Second, they must express CD105, CD73 and CD90, and lack the expression of CD45, CD34, CD14 or CD11b, CD79a or CD19 and HLA-DR surface molecules. Third, they must differentiate to osteoblasts, adipocytes or chondroblasts *in vitro* [4]. However, in contrast with stem cells from different sources, the immunophenotype of MMSCs is far from being completely clarified, and discrepancies among different species, tissue sources or methods of analysis are identifiable in the scientific literature [5]. Moreover, heterogeneous populations with different levels of stemness obscure this concept and the identity of these cells in *in vivo* environments is not yet clear [6].

In a recent ultrastructural study of cultured equine adipose-derived MMSCs, the authors discussed that most previous studies have been focused on isolation, *in vitro* characterization and immunophenotyping of these cells, but not on establishing their ultrastructural pattern [5].

As an ultrastructural "gold-standard" for MMSCs identification in humans is clearly needed, we have developed a protocol to properly identify it.

Materials and Methods

Cell cultures

Human raw tumescent lipoaspirate was washed thoroughly with phosphate-buffered saline (PBS) containing 250 U/mL Penicillin, 200 µg/mL Streptomycin, 500 µg/mL Neomycin and 10 µg/mL Amphotericin β to remove contaminating debris and red blood cells. The washed sample was treated with 1 mg/mL Collagenase (type I, Sigma-Aldrich) in PBS for 40 minutes at 37^0C in a shaking water bath. After the enzymatic digestion,

the sample was centrifuged for 10 minutes at 500 g and the cell pellet was suspended in Dulbecco's Modified Eagle Medium (DMEM, Sigma-Aldrich) containing 10% fetal bovine serum (FBS), and filtered trough a 100-µm mesh to remove remaining debris. The filtrate was washed 2× in DMEM-10% FBS and plated onto conventional cell culture flasks. After 48 hours, the medium was discarded; the adherent cells were washed once with PBS, and fresh DMEM-10% FBS was added.

Flow cytometry

The cells were seeded at a density of 5×10^4 and left 48 hours to adhere and divide. A BD LSR II flow cytometer (BD Biosciences) was used to perform the analysis of the cultures. The culture medium was discarded, the cells were harvested in 0.25% Trypsin/EDTA and fixed for 30 minutes in ice-cold 2% formaldehyde. The fixed cells were washed 2× in PBS and incubated for 30 minutes in 350 µL PBS containing the FITC (Fluorescein Isothiocyanate) or PE (Phycoerythrin) labeled antibodies (BD Biosciences), specific for CD73, CD90, CD105, CD34 and CD45. Unstained cells were used as control, to assess their autofluorescence.

Electron microscopy

For transmission electron microscopy (TEM) analysis, cells were seeded at a density of 1×10^5 cells/mL in a cell culture plate and left two days to reach confluence. A confluent culture plate with human adipose-derived MMSC was fixed with 2.5% Glutaraldehyde for 10 minutes, post-fixation was done using OsO_4 for 10 minutes. Tannic acid was used for staining. The cells were dehydrated in a graded series of ethanol up to absolute. Epon embedding was done en-face, using Epon filled Eppendorf 0.5 mL tubes laid up side down on the cell monolayer; after polymerization, the tubes were carefully broken off, containing the intact cells. Ultrathin sections were cut using a Leica EM UC6 ultramicrotome, the sections were mounted on 400 mesh copper grids and examined with a Philips EM 208 transmission electron microscope using a Veleta video camera and the iTEM Olympus Soft Imaging System.

Results

Flow cytometry

The flow cytometry evaluation of surface markers resulted in an expected profile (Figure 1): the obtained cells expressed CD73, CD90 and CD105, and were negative for CD34 and CD45. The population of interest was gated on a FSC/SSC dot plot (Figure 1A).

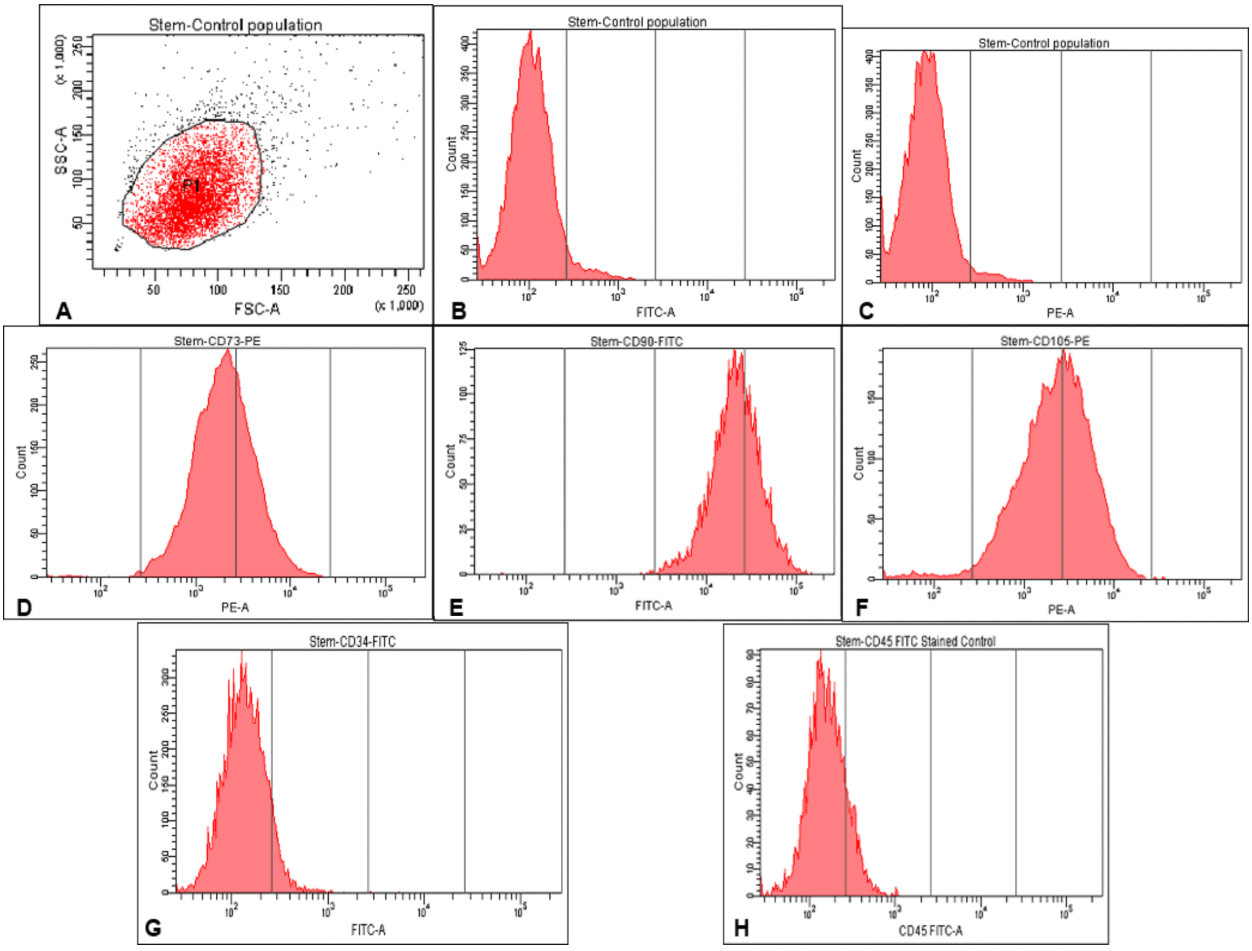

Figure 1 – ***Flow cytometry results. (A–C) Control cells, used for parameter setup and population gating. (D–F) Cytometry histograms for CD73, CD90 and CD105, the fluorescence peak is clearly displaced to the right compared to the control sample indicating that the cells are positive for these three markers. (G–H) Results for CD34 and CD45 staining, the FITC fluorescence peak did not move significantly compared to the control tube, indicating that the cells do not express CD34 and CD45 on their surface.***

TEM

The TEM examination of the MMSC revealed the following set of ultrastructure features: the cell body was either rounded, oval or polygonal in section and the nuclei were eccentric, large, pale, euchromatic, with 1–2 nucleoli, peripheral heterochromatin, with frequent nuclear clefts and invaginations of the nuclear envelopes (Figures 2 and 3).

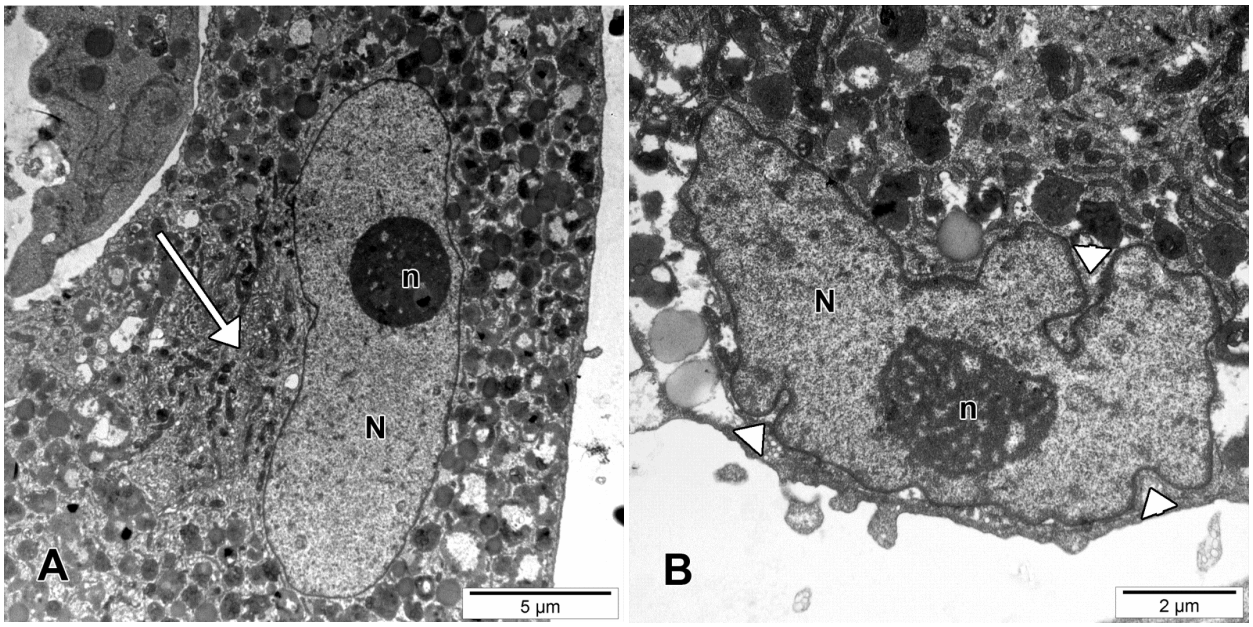

Figure 2 – ***The general aspect of MMSCs in TEM examinations. Nuclei (N) may present regular outline (A) or may be indented (arrowheads, B), and may have visible nucleoli (n). Mitochondria (arrow, A) are identified, but not exclusively in a juxtanuclear area.***

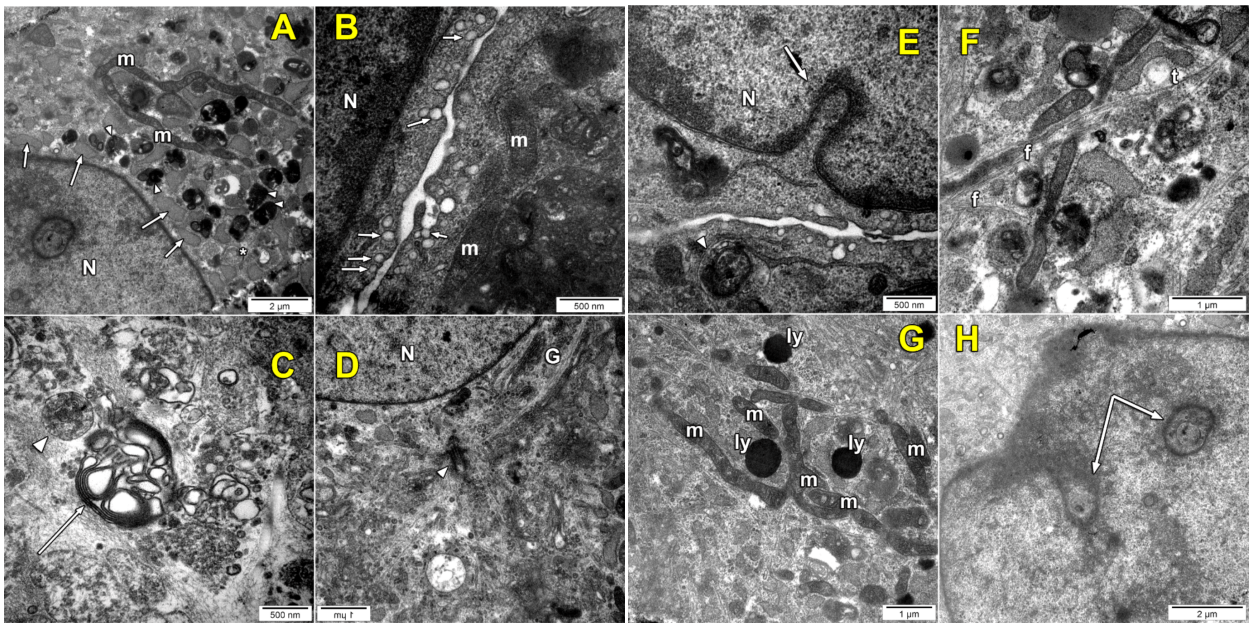

Figure 3 – ***Ultrastructural details of human MMSC. (A) N – Nucleus; m – Mitochondria; arrows – Dilated cisterns of endoplasmic reticulum; arrowheads – Residual bodies. (B) Two neighbor MMSC display several large subplasmalemmal caveolae (arrows); m – Mitochondria; N – Nucleus. (C) A multivesicular body (arrowhead) and myelin figures (arrow) are identified. (D) In the vicinity of the nucleus (N), the Golgi apparatus (G) and a centrosome (arrowhead) are identified. (E) Nucleus (N) of a MMSC displaying a nuclear cleft (arrow); the neighbor cell presents a multivesicular body (arrowhead) with electronodense content. (F) Bundles of intermediate filaments (f) and long microtubules (t) cross within the cytoplasm, and intermingle with various organelles. (G) Long mitochondria (m) and lysosomes (ly). (H) Nuclear clefts (arrows), in different incidences.***

The cell membranes were irregular and small protrusions were frequently identified; rarely, subplasmalemmal caveolae were found, mostly in cell regions with poor content of perinuclear cytoplasm (Figure 3). A peculiar pattern of the organelles was observed (Figures 3 and 4): (a) abundant ribosomes and polyribosomes that were ubiquitous throughout the cytoplasm; (b) the rough endoplasmic reticulum (RER) consisted of: (*i*) extremely numerous large and dilated cisternae with moderately dense granular contents, and (*ii*) rare long,

thin and irregular cisternae; (c) a high concentration of long mitochondria (up to 4–5 μm) that were frequently grouped with dilated cisternae of RER and endosomes; the dense mitochondrial matrix was a constant feature; (d) the Golgi apparatus was well developed, consisting of numerous flattened cisternae and vesicles. Lysosomes were scarce. Residual bodies were particularly numerous. Lipid droplets were rarely detected. Secretory granules were also distinguished. Endocytic vesicles were numerous within the cytoplasm; many of these were present in subplasmalemmal regions and were generated as coated pits. Endosomes were frequently detected: early endosomes, multivesicular bodies (MVBs), endolysosomes and autophagosomes. The cytoskeleton (Figures 3 and 4) presented: (a) long and numerous microtubules, occasionally grouped, randomly dispersed in the cytoplasm; (b) centrosomes were occasionally identified; (c) intermediate filaments were usually grouped in bundles in the cytoplasm; moreover such bundles were particularly distributed beneath the cell membrane, lining it and ending deeper within the cytoplasm.

We also identified two types of cell prolongations, thick and thin. The thick cell processes (Figures 4 and 5) had a peculiar structure: one side was filaments-rich, with consistent subplasmalemmal bundles of filaments, while the opposite side was devoided of filaments and rather vesicular, with coated-pits and clathrin-coated vesicles; rare endosomes and microtubules were identified within the axial core of these processes. The thin, slender and dendritic processes (Figure 6), were mostly built by filaments extending from the cell membrane subsurface; these long and dichotomous (filopodes) (Figure 7) and short (microvillar) cell prolongations arose either solitary or grouped, and were interconnected or connected to neighbor cells and processes. These filopodia were also found as collaterals branches emerging from the thick cell processes. We also occasionally identified such filopodia displaying dilations containing either mitochondria and secretory granules (Figure 6) or endocytotic vesicles.

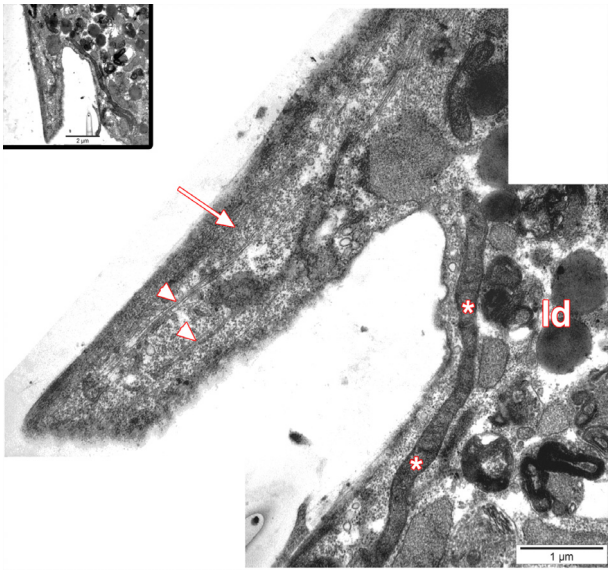

Figure 4 – ***Ultrathin section through a MMSC, at the emergence of one of its processes: inset – general view, at low resolutions; the main figure is a reconstructed image. Subplasmalemmal filaments are grouped (arrow) on the outer side of the process, which is also rich in microtubules (arrowheads). Within the cell body lipid droplets (ld) and a long mitochondria (*) are identified.***

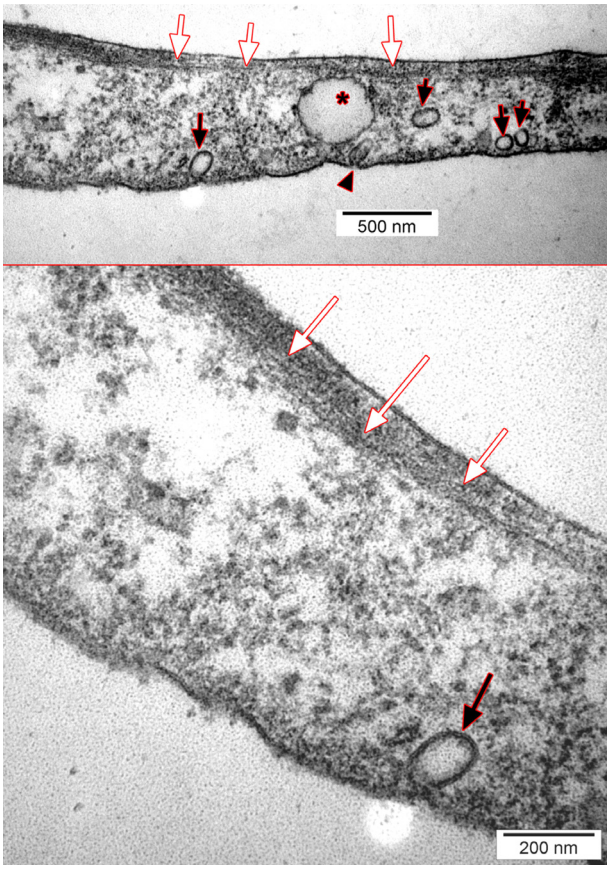

Figure 5 – ***Detailed ultrastructure of a MMSC process. Characteristic asymmetrical disposition of cytoskeletal filaments on one side of the process (white arrows) that seemingly block any transmembranar vesicular transfer. Clathrin-coated pits (arrowhead) and subplasmalemmal clathrin-coated vesicles (black arrows) are associated with the opposite sides of the processes. In the upper panel, a late endosome is indicated (*).***

Discussion

Human adipose tissue is largely recognized as a potential source of stem cells for regenerative medicine applications [7–18]. The human adipose-derived MMSCs we found had fulfilled the ISCT standards of identification. The adipose tissue we used was gathered by tumescent liposuction. This is in accordance with a study showing that the yield and growth characteristics of adipose derived stem cells are affected by the type of surgical procedure used for adipose tissue harvesting; resection and tumescent liposuction seem to be preferable compared to ultrasound-assisted liposuction for tissue-engineering purposes [19].

MMSCs can be found *in situ* within the supportive stromal compartment of resident tissues [3]. For example, human stromal cells isolated from skin have similar immunophenotype to bone marrow and adipose-derived MMSCs and could be differentiated into adipogenic and osteogenic lineages under the proper induction conditions [20]. These data suggest the need for a clear distinction in TEM between MMSCs and other stromal cell types.

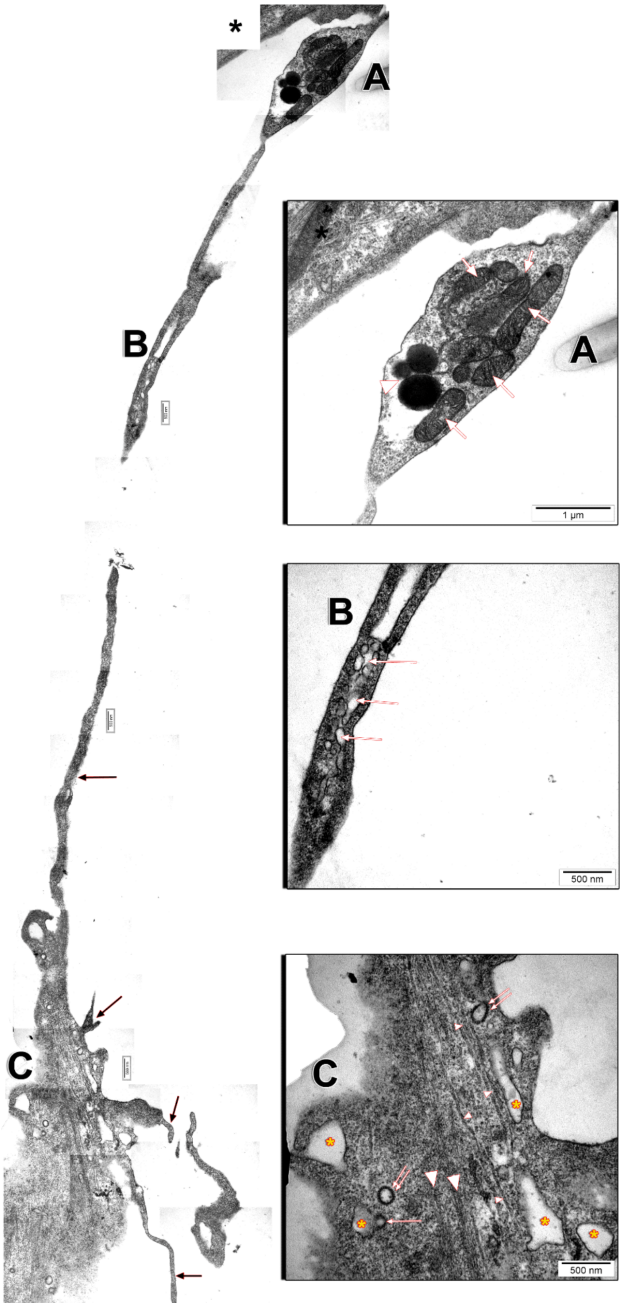

Figure 6 – ***Ultrathin section, human adipose-derived MMSC, reconstructed image (left side) and details (A–C insets, right side). The long and slender MMSC processes are indicated (reconstruction, black arrows). In (A), a proximal dilatation of a MMSC process is depicted, containing a large number of elongated mitochondria (arrows) and secretory granules (arrowhead). In (B), numerous endocytotic vesicles within a MMSC process fuse with an early endosome. In (C), numerous late endosomes are evidenced (*) as also are clathrin-coated pits (thin arrow) and vesicles (doubled thin arrows), bundles of intermediate filaments (thick arrowheads) and numerous microtubules (thin arrowheads).***

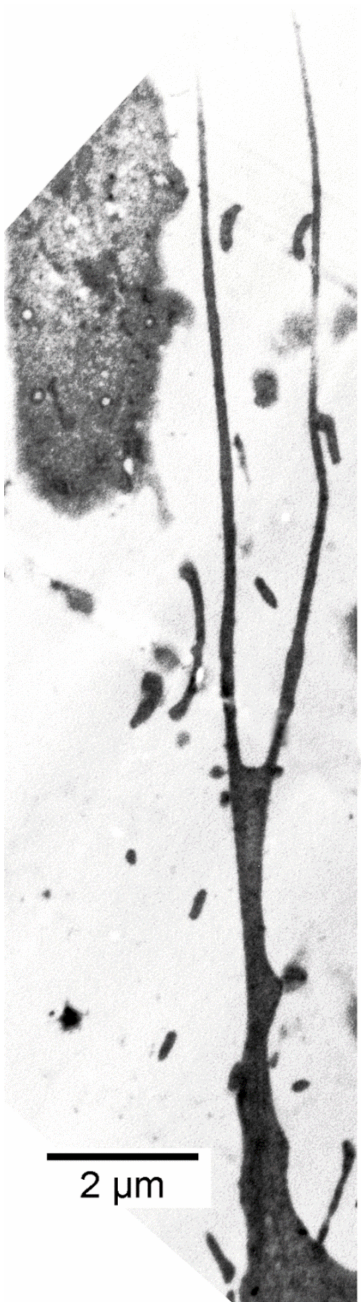

Figure 7 – ***Dichotomous long and thin prolongation (filopode) of a MMSC.***

As MMSCs do not meet general criteria for stem cell activity [3], markers, such as CD117/c-kit, which labels germ cells and stem cells, could be improper for their identification. MMSCs are also negative for CD34 [4]. In this regard, the observation of Díaz-Flores *et al.* needs attention, these authors commenting recently that although the CD34-positive stromal fibroblastic/fibrocytic cells are given different and confusing names in various studies they are in fact a distinctive subset of fibroblasts in perivascular and stromal positions within tissues, that behave as native mesenchymal stem cell progenitors after losing the CD34 expression [21].

A reliable tool to distinguish between MMSCs and other stromal cell types *in situ* is electron microscopy,

and this technique should be used as the "gold standards" differentiating various cells from that niche.

Our study bridges the interspecies gap by confirming that the ultrastructure features of the equine adipose-derived MMSC [5] are also identifiable in human MMSCs. An ultrastructural pattern of MMSCs, if not a "gold standard", of identification, may be postulated, and referred to, in further TEM studies although it was previously stated [22] that "defining mesenchymal stem cells *in vitro* adds complexity to their study because the artificial conditions may introduce experimental artifacts". The biological effects of the equine adipose-derived MMSCs are related to a paracrine mechanism through membrane vesicles, including shedding vesicles and exosomes [23].

The most striking features of the MMSCs are the extremely rich content of endosomal/vesicular elements, long mitochondria and extremely dilated RER cisternae. The cytoskeletal pattern of the MMSCs is also rather specific. There are not available in our knowledge other TEM studies of human MMSCs resulted from *in vitro* conditions.

In addition to the common features of the equine and human adipose-derived MMSCs, we were able to show that, in humans, MMSCs prolongations are of two types: (a) thick processes, with a filaments-rich side opposed to a rather vesicular one, and (b) slender processes (filopodes and pseudopodes), filaments-rich, building labyrinthine systems. We also proved that the filopodes of MMSCs present dilations accommodating mitochondria, secretory granules, and vesicles. The morphology of these filopodes is consistent with the description of the telopodes (Tps), which are the prolongations of telocytes (TCs).

However, ultrastructure differences should be accounted among MMSCs of various origins, despite a similar immunophenotype; this was proven for MMSCs derived from human bone marrow and term placenta: (a) bone marrow MMSCs had mesenchymal features with dilated cisternae of rough endoplasmic reticulum and peripheral collections of multiloculated clear blisters mostly representing complex foldings of the plasma membrane; (b) chorion term placenta MMSCs were more primitive and metabolically quiescent, their major features being the presence of rough endoplasmic reticulum stacks and large peripheral collections of unbound glycogen, and (c) amnion term placenta MMSCs showed a hybrid epithelial-mesenchymal ultrastructural phenotype; epithelial characters included non-intestinal-type surface microvilli, intracytoplasmic lumina lined with microvilli, and intercellular junctions; mesenchymal features included rough endoplasmic reticulum, lipid droplets, and well-developed foci of contractile filaments with dense bodies [24].

Comparing plastic-adherent cells with tissues-resident stem cells is a very difficult task as also is to decide what actually is a cell identified through a specific methodo-logy: a stem cell, an early transit-amplifying cell (TAC), a late TAC, or a differentiated progeny. In this regard, cells lacking a distinctive excretory apparatus could be in fact TACs, and this could justify the phenotypic differences between different reports.

TEM can be a reliable tool to be used to clarify and pattern the various classes and subclasses of stromal cells, which is extremely important for regenerative processes and tissue remodeling follow-up. However, further studies should evaluate the *in vivo* stem niches to check whether or not the *in vitro* ultrastructure pattern of MMSCs could correspond to an *in situ* one.

Author contribution

All the authors have equally contributed to this study.

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