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

Explants-isolated human placenta and umbilical cord cells share characteristics of both epithelial and mesenchymal stem cells

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Abstract

In recent years, identification of new sources of adult stem cells developed rapidly, pursuing to find easily available tissues, which will give rise to homogenous stem cells populations. Up to present, bone marrow-derived mesenchymal stem cells (BM-MSCs) are unanimously considered to fulfill the criteria for being used in clinical settings, but adipose stem cells, placental and umbilical cord stem cells, and other tissue-derived stem cells are making their way to being used at least in autologous transplantation. We isolated cellular populations from placental tissue and umbilical cord using the explants method. The placental (PL) and umbilical cord (UC)-derived cells were cultured and expanded in appropriate conditions for generation of stem cells. We assessed the stemness characteristics of the tissue-isolated cells and compared them to an established MSCs line. For this purpose, we determined the immunophenotype, morphological and ultrastructural characteristics, as well as functional abilities of PL- and UC-derived cells. Flow cytometric evaluation of cells revealed presence of CD90, CD73, and CD105 stem cells markers, while the cells were negative for CD34, CD45 and HLA-DR. Immunocytochemical staining showed that 100% of PL- and UC-derived cells are positive for vimentin and CD105 expression, while cytokeratin was revealed in less than 10% in both tissue-isolated cells. Morphological and ultrastructural characteristics of cells exposed analogous cellular size and intracellular organization, similar to MSCs, but detailed view of UC-derived cells by transmission electron microscopy (TEM) demonstrated presence of intercellular junctions–desmosomes, similar to epithelial cells. Both PL- and UC-derived cells confirmed their trilineage potential, being able to differentiate into adipocytes, osteoblasts, and chondrocytes in different proportions. Flow chamber *in vitro* assay was used to determine to what extent PL- and UC-derived cells are able to adhere to substrates (VCAM and ICAM) and we showed progressively decreased adhesion of both cellular types, inversely proportional to the generated shear stress. We may conclude that explants-isolated placental and umbilical cord cells are endowed with characteristics of both epithelial and mesenchymal stem cells, and purification procedures are additionally required for safe use of these cells in diverse clinical applications.

Keywords: placenta-derived cells, umbilical cord-derived cells, immunophenotypical characteristics, differentiation, adhesion behavior.

Introduction

After isolation of the first embryonic human stem cell, in 1998 [1], ethical considerations and tumor formation [2] have limited and restrained scientific research on these cells. Much of the attention has shifted to exploring adult stem cells, particularly mesenchymal/stromal stem cells (MSCs). Bone marrow-derived mesenchymal stem cells remain the most extensively studied cellular type. However, harvesting stem cells from the bone marrow requires an invasive procedure, being isolated from the bone marrow of superior iliac crest, while MSCs from the femur or tibia during hip or knee replacement surgery are a suitable alternative [3]. Human term placenta has recently emerged as a novel source of MSCs [4, 5]. Placenta, as a medical waste, is usually discarded without any ethical conflict. This fetal tissue is considered a good easily available MSCs source for stem cell therapy and banking of stem cell populations. In this context, cells phenotypically similar to bone marrow-derived MSCs have

been isolated from various extra-embryonic structures, including amniotic fluid, Wharton's jelly, amniotic and chorionic membrane [5]. The MSCs from these sources are easy to obtain and also cultured at low cost. MSCs not only participate in the regeneration of tissues of mesenchymal lineages [6–8], but also appear to differentiate into cells derived from other embryonic layers, including skin [6] and lung [9]. These experiments demonstrate the plasticity of MSCs and their potential usefulness in complex tissue repair and regeneration, and in cell therapy. It has also been suggested that MSCs may be able to affect immune responses in the body to reduce inflammation and help treat transplant rejection or auto-immune diseases and can contribute to skin reconstitution in cutaneous wounds [10–12]. In addition, neither autologous nor allogeneic MSCs induce immunoreactivity in the host on local transplantation or systemic administration [13, 14]. Thus, these cells represent an important building block for cell therapy, wound repair, regenerative medicine, and tissue engineering [15].

Initially, MSCs were selected by adherence to the plastic surface and were thought to consist of a homogenous population, but recently, more MSCs markers have been identified that are suitable to show heterogeneity of these cells [16–18]. Although many of these markers are very selective in their recognition of MSCs, they lack the ability to discriminate between MSC subsets [19, 20], while the isolated populations show distinct morphological features and differentiation capacities [21–23]. In 2006, the *International Society for Cellular Therapy* proposed the minimal criteria to define MSCs [24], which unified the concept of mesenchymal stem cells and provided easy identification of MSCs subsets. From the published data, placental tissue is an important source of stem cells, and additional useful cell types in this tissue may yet be defined, each with variable phenotypical characteristics and plasticity [25].

The aim of the present study was to evaluate the placenta and umbilical cord-derived cells from the immunophenotypical point of view, and to conclude to what extent these cells are comparable to bone marrow-derived MSCs. Further on, we tested the adhesion potential of these cells for better understanding of the behavior pattern that governs cellular migration towards injured sites, in the view of their use in cellular therapies.

⊟ Materials and Methods

Isolation of cells from human placenta and umbilical cord

Placental and umbilical cord samples were obtained from pregnant female patients submitted for term delivery, either vaginal or cesarean section (*n*=15), randomly selected from maternal or fetal sites. Usually, both placenta and umbilical cord are removed and discarded, becoming medical waste, unless other procedures are already contracted by the patients. Placental (PL) and umbilical cord (UC)-derived cells were isolated by explants method, and cells were cultured and expanded in alpha-minimum essential medium (MEM; Gibco BRL, Carlsbad, CA, USA), supplemented with 10% Fetal Calf Serum (FCS; PromoCell, Heidelberg, Germany) and 1% Penicillin/Streptomycin solution (10 000 IU/mL; PromoCell, Heidelberg, Germany). Cells were used in these experiments at passages 2–5. All tissue samples were obtained after signing the written informed consent elaborated under an approved protocol by the Ethics Committee of "Victor Babeş" University of Medicine and Pharmacy, Timişoara, Romania, according to the *World Medical Association Declaration* of Helsinki.

As control cells, we used cord blood-derived human mesenchymal stem cells line (MSCs; SC00A01, Vitro Biopharma, CO, USA), cultured in MSC-Gro™ Growth Media, according to provider specifications.

Transmission electron microscopy

Transmission electron microscopy (TEM) was used to define MSCs' ultrastructural characteristics. For TEM analysis, cells were processed using the method previously described by authors [26]. Digital electron micrographs were recorded with a MegaView III CCD using iTEM-SIS software (Olympus, Soft Imaging System GmbH, Germany).

Immunophenotypical profile of placenta and umbilical cord-derived cells

Flow cytometry analysis

Cultured cells (PL, UC, and MSCs) reaching 80–90% confluence were detached from the culture plate, washed and stained with mouse anti-human conjugated antibodies following the manufacturer instructions (BD Pharmingen™, Heidelberg, Germany). Phycoerythrin (PE)-conjugated – CD73, fluorescein isothiocyanate (FITC)-conjugated – CD34, CD90, CD105, and HLA-DR, and allophycocyanin (APC)-conjugated antibodies – CD45 were used for data acquisition on FACSCalibur flow cytometer (BD Biosciences), while analyses were performed using Flowing Software 2.5.1 dedicated software.

Immunocytochemical/immunofluorescence analyses

MSCs, PL- and UC-derived cells prepared for these analyses were fixed with methanol and investigated for expression of interest markers, employing mouse anti-human antibodies for vimentin (clone V9), endoglin, CD105 (clone SN6h), Ki67 antigen (clone MIB-1), and cytokeratin (clone MNF116). All primary antibodies were provided by DakoCytomation (Glostrup, Denmark), and tested for human specificity and cross-reactivity. Staining protocol continued with secondary biotinylated antibody binding, substrate addition, and Hematoxylin counter-staining of the nuclei [DakoEnVision Systems–horseradish peroxidase (HRP)] following the manufacturer procedures. Vimentin expression was revealed by fluorescence method, after coupling the primary antibody with specific fluoro-chrome-conjugated secondary antibody (AlexaFluor 594, Invitrogen™, Carlsbad, CA, USA).

Adhesion potential assay of placenta and umbilical cord-derived cells

Fluid Shear Stress assay was used for investigation of adhesion potential of MSCs, PL- and UC-derived cells to ICAM (intercellular adhesion molecule) and VCAM (vascular cell adhesion molecule) substrates, at a concentration of 2 μg/mL. The assay employed a peristaltic pump (IPC 8 Ismatec, Germany) and 10^5 suspension cells in Hank's buffered salt solution (HBSS) medium (Sigma-Aldrich Company, St. Louis, Missouri, USA) were added on each substrate-coated channel of six channel μ-Slide (Ibidi, Germany). The cells were exposed to progressively increasing shear stress of 0.35, 2, 5, 8 and 15 dyne/cm^2, and serial microphotographs were taken for each value, cell number being compared with the initial adherent cells.

Differentiation potential

MSCs, PL- and UC-derived cells were tested for their *in vitro* trilineage potential, using specific in-house made differentiation media for generation of adipocytes, chondrocytes, and osteoblasts. Adipogenic differentiation was revealed 21 days after medium induction, and lipid

vacuoles were stained with Oil Red O (Sigma Aldrich Company). Chondrocytes appeared in culture flasks 24 days after induction and were stained for aggrecan (R&D Systems, Minneapolis, MN, USA), while osteogenic differentiation was unveiled by osteocalcin staining (R&D Systems), 10 days after specific medium addition.

⊟ Results

Placenta and umbilical cord-derived cells share similar immunophenotypical characteristics with MSCs

As a result of flow cytometric assessment, we found that placenta and umbilical cord-isolated cells are negative (<1%) for membrane markers such as CD34, CD45, and HLA-DR, similar to control MSCs and literature data. Expression of CD90 presented variation between different samples, more than 95% of placenta-derived cells presenting this marker on their surface, while umbilical cord cells expressed CD90 in a proportion of 88.69%. CD73 presented similar pattern of expression to CD90, 94.86% of PL cells, and 95.71% of UC cells exhibiting this marker. Another marker required for positive identification of mesenchymal stem cells is CD105, which was expressed on the average 65.50% of PL-derived cells and 64% of UC-derived cells. This low expression of CD105 detected by flow cytometry can be related to cellular processing procedures during this type of assessment, which require the use of proteolytic enzymes (trypsin) and may induce internalization of the surface epitope (Figure 1). Other surface molecules were tested for their presence on PL- and UC-derived cells, thus being able to identify CD44 (89.23% and 47.7%, respectively) and CD29 (98.57% and 96.7%, respectively) (data not shown).

When assessed by immunocytochemical methods, CD105 (endoglin) was revealed in 100% cells, thus confirming the mesenchymal stem cells characteristics of both placenta and umbilical cord-derived cells. Proliferation rate was evaluated by presence of Ki67 nuclear marker at the level of PL and UC cells at different passages (from passage 2 to passage 5), counted on five different microscopic fields and resulted in an average 29.8%, 34.8% and 35.2% proliferation rates for MSCs, PL- and UC-derived cells, respectively, with no significant difference between cellular populations considered for this study (Figure 2).

We performed immunofluorescence on MSCs, PL- and UC-derived cells for vimentin, another marker associated with cytoskeleton of mesenchymal stem cells. All cellular types were 100% positive for vimentin fibers, as shown in Figure 3, thus demonstrating they can be included in the mesenchymal stem cells cluster.
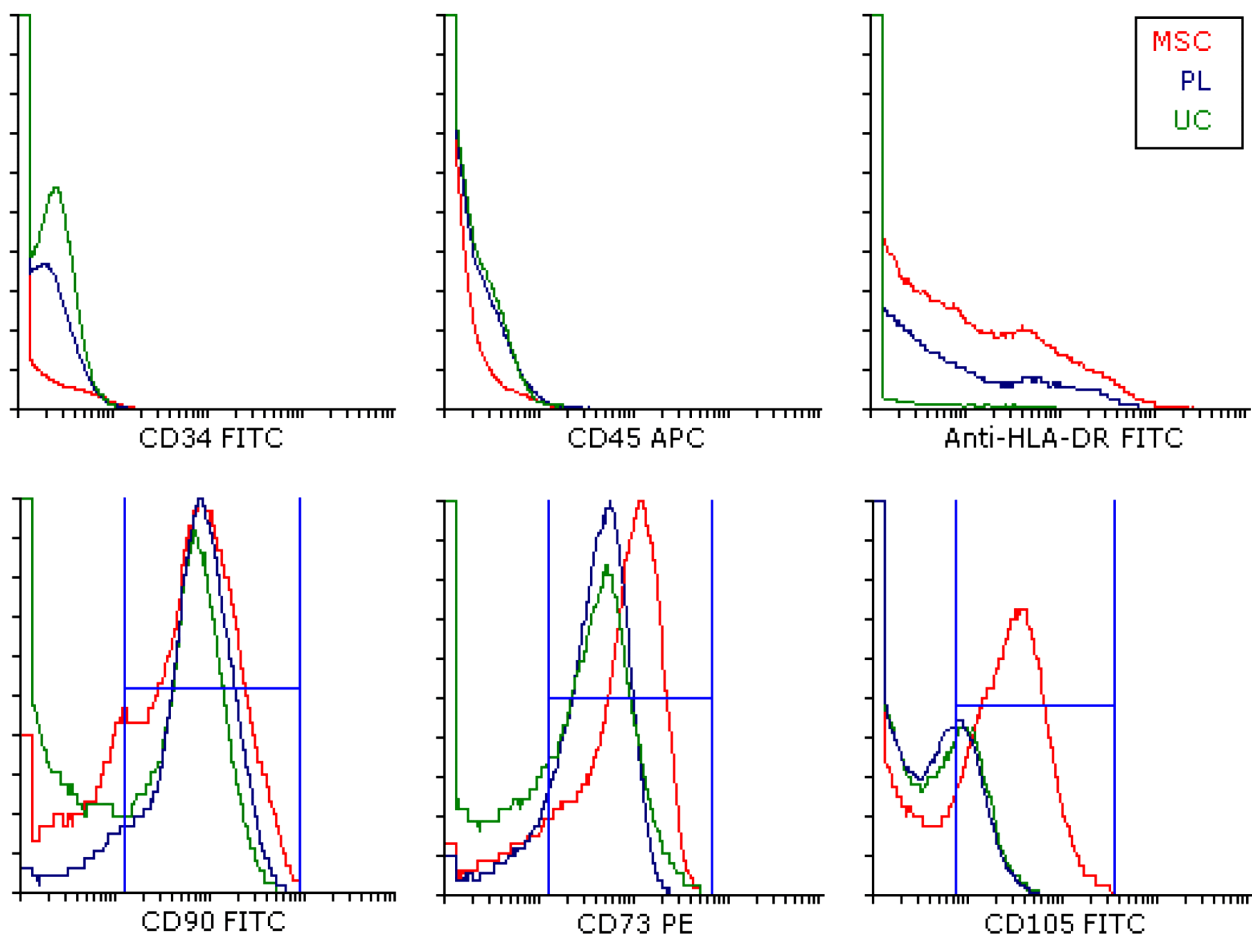

Figure 1 – *Expression of phenotypical markers at the level of cell membrane in MSCs, PL- and UC-derived cells. Cells are negative for CD34, CD45, and HLA-DR, while showing positive expression of CD90, CD73, and CD105. Similar pattern of expression between all three cellular types.*
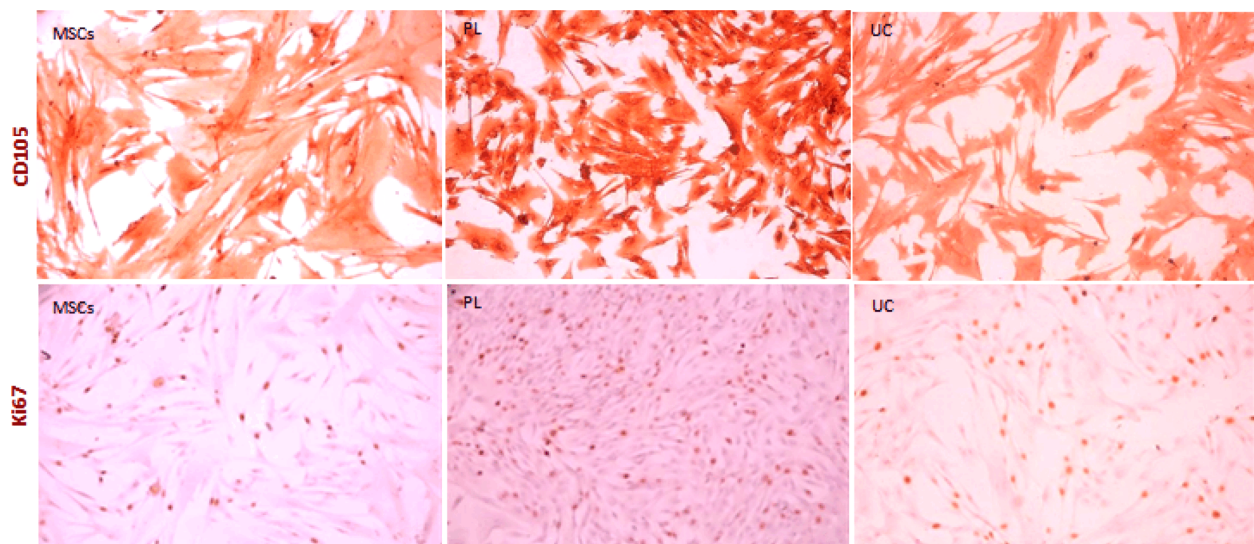
Figure 2 – *Immunocytochemical evaluation of stem cell markers. CD105 is expressed by all cellular types (upper panel, ×200), while proliferation rate, reveled by Ki67 presence at the nuclear level, showed that almost one-third of the cells are multiplying (lower panel, cells at passage 4, ×100).*
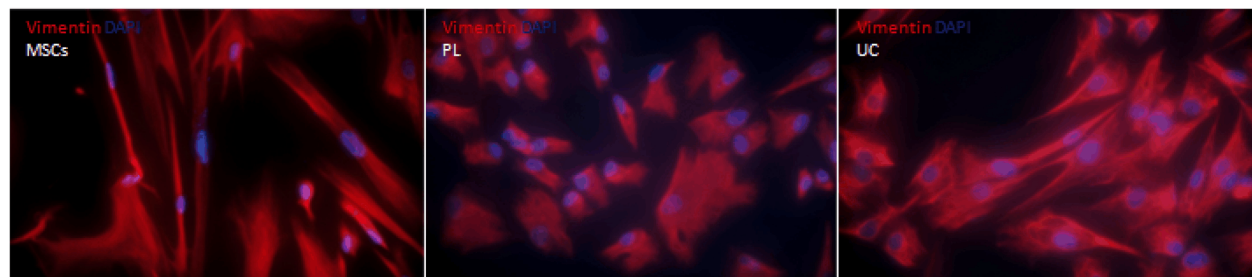

Figure 3 – *Positive vimentin expression in MSCs, PL- and UC-derived cells. From the morphological point of view, different cell sizes can be seen in tissue-isolated samples, both placenta and umbilical cord (×200).*

Placenta and umbilical cord-derived cells exhibit epithelial features

In optic microscopy, all cellular types were adherent to plastic culture flasks and shared a similar fibroblast-like morphology, with slight variation in cells size (data not shown). When analyzed in transmission electron microscopy (TEM), the average cell diameter was determined, being between 10–15 μm for MSCs, PL- and UC-derived cells. No significant differences were revealed regarding the ultra-structural cytoplasmic or nuclear components. However, detailed and enlarged view of UC-derived cells showed the presence of intercellular junctions–desmosomes (Figure 4), thus demonstrating the epithelial-like features of these cells. We were not able to identify similar types of inter-cellular junctions in MSCs or PL-derived cells.

Immunocytochemical staining of MSCs, PL- and UC-derived cells gave further support for the epithelial-like characteristics of these cells, when cytokeratin presence was detected in all cellular types in different proportions. UC-derived cells expressed cytokeratin in 10% of the cells, counted on five different microscopic fields, while MCSs and PL-derived cells expressed this marker in less than 2% of the cells (Figure 5).

Placenta and umbilical cord cells are able to differentiate *in vitro* towards adipocytes, chondro-cytes, and osteoblasts

We performed differentiation of MSCs, PL- and UC-derived cells towards mesodermal cell lineages –

adipogenic, chondrogenic, and osteogenic. Similar to MSCs, both PL- and UC-derived cells are endowed with *in vitro* trilineage differentiation potential, but there are differences between the differentiation rate, depending on cellular type and cellular lineage they are induced into. Surprisingly, adipogenic differentiation had the highest rate in UC-derived cells (more than 80%), compared to MSCs (50%) and PL-derived cells (42%) (Figure 6, Oil Red O staining). Aggrecan immunocytochemical staining revealed the chondrogenic differentiation ability of the cells, showing similar results for all cellular types (approximately 70% of the cells), which were induced *in vitro* in monolayer cell culture (Figure 6, Aggrecan staining). Osteocalcin was used as marker of osteogenic differentiation and was present in 50% of MSCs, 35% of PL-derived cells, and 80% of UC-derived cells (Figure 6, Osteocalcin staining).

Analogous pattern of adhesion behavior to VCAM and ICAM

For further functional studies, we used the flow chamber *in vitro* assay to determine to what extent PL and UC-derived cells are able to adhere to substrates (VCAM and ICAM coated slide channels), when a pro-gressively increasing shear stress was generated (from 0.35 to 15 dynes/cm^2). VCAM presented as a better molecule binding MSCs, as well as PL- and UC-derived cells. MSCs progressively detached from the substrate as the shear stress increases, so that at 15 dynes/cm^2, only 57% of the cells remain attached. Adhesion of PL- and UC-derived cells to VCAM ligand was even lower

compared to MSCs, for the same values of induced shear stress, at 15 dynes/cm^2, only 22% and 25% of initially adherent cells were counted on chamber slide for PL and UC, respectively. ICAM adherence and adhesion of cells was lower compared to VCAM molecule, for the same generated shear stress. Only 54% of MSCs, and 22% of PL- and UC-derived cells remained attached when 15 dynes/cm^2 shear stress was generated (Figure 7).
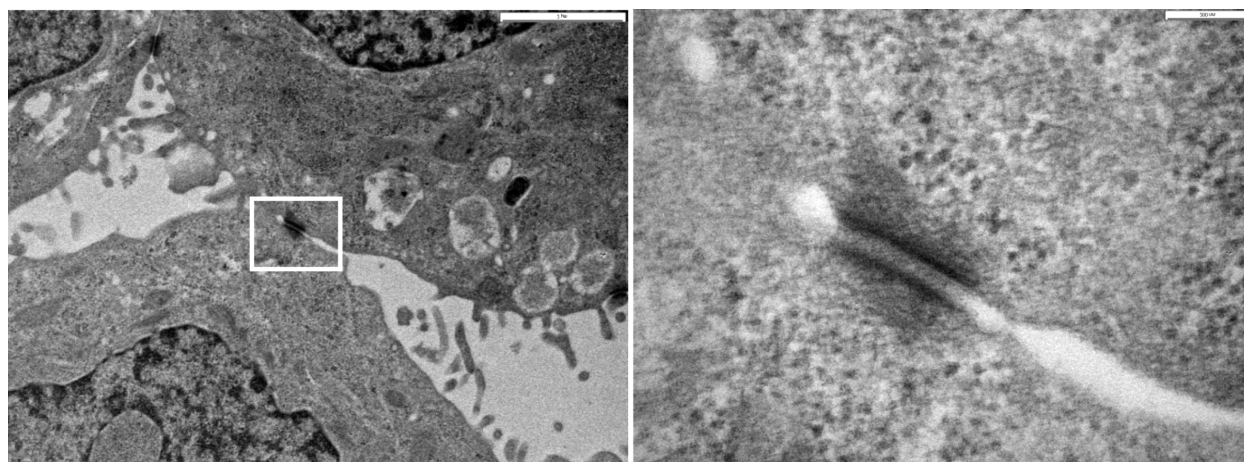

Figure 4 – *Ultrastructural details of UC-derived cells revealed by TEM. Presence of desmosomes connecting two individual UC-derived cells (left panel) and enlarged view of intercellular junction (right panel).*
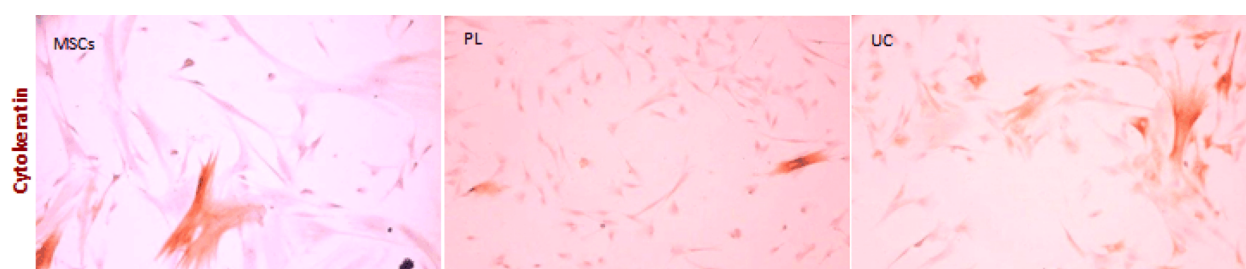

Figure 5 – *Cytokeratin expression. Variable expression of cytokeratin in all cellular types (×200).*
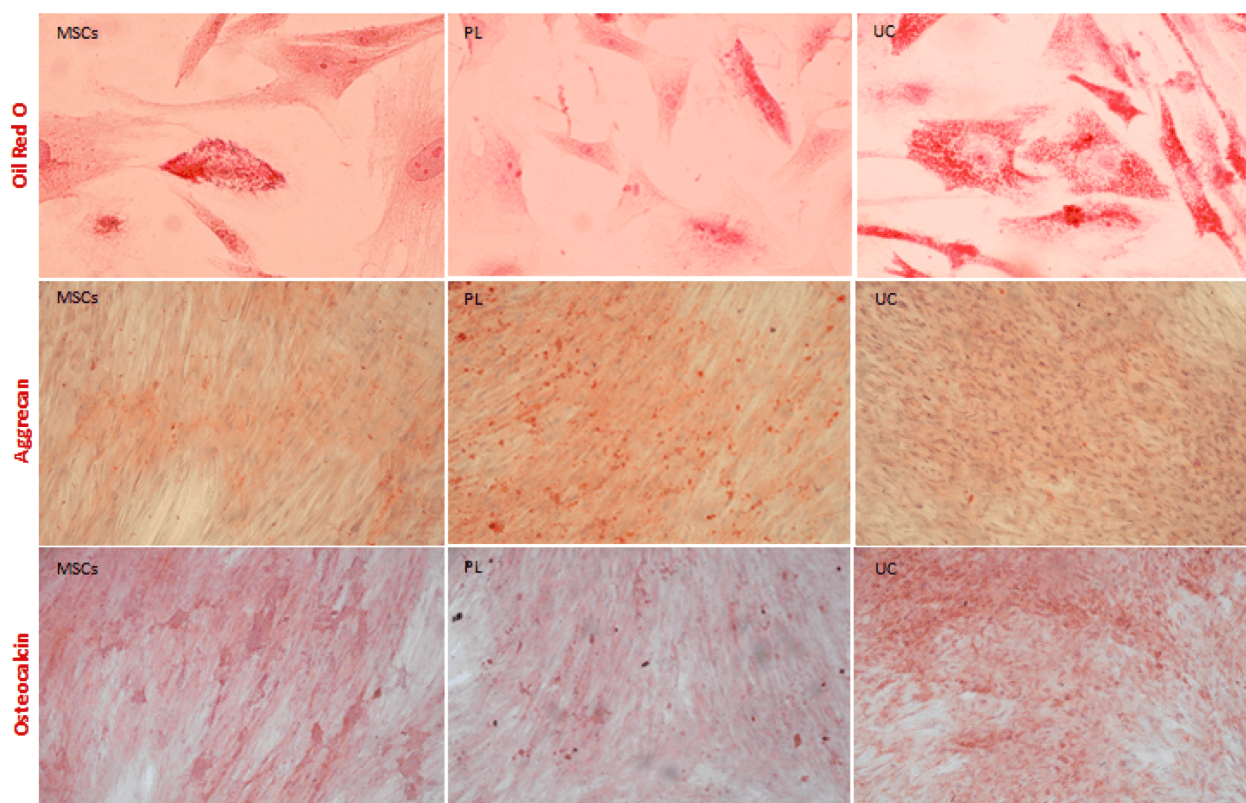

Figure 6 – *Trilineage differentiation potential of MSCs, PL- and UC-derived cells. All cellular types are endowed with the ability to differentiate into adipocytes (Oil Red O staining, ×400), chondrocytes (Aggrecan staining, ×100), and osteoblasts (Osteocalcin staining, ×100), when are induced in vitro with appropriate culture media, but there are differences between the ratio at which this process is performed.*
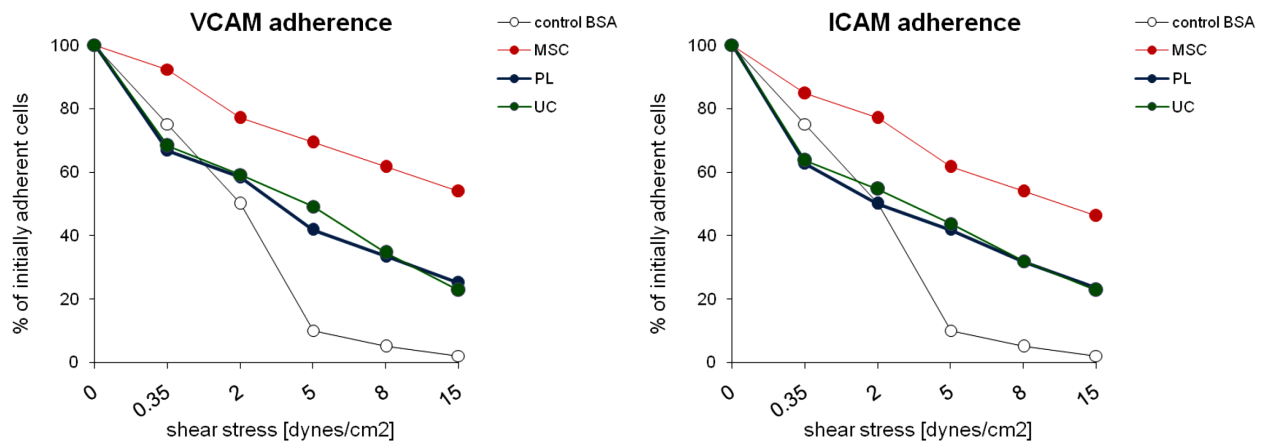
Figure 7 – *Flow chamber assay for VCAM and ICAM. Comparable pattern of adhesion behavior is shown between PL- and UC-derived cells, for both VCAM and ICAM ligands, but with lower values than in MSCs at the same shear stress points.*

⊟ Discussion

Definition of adult stem cells is evolving as the research is adding more information about their functionality and morphology, but most researchers agree that adult stem cells are isolated from postnatal organism, from tissues of endodermal, mesodermal and ectodermal origin, manifest pluripotent markers and do not form teratomas [27]. Mesenchymal stem cells (MSCs) were first identified in bone marrow of adult subjects (the "other" bone marrow stem cells) and subsequently studies identified MSCs in other tissues like adipose, cartilage bone, muscles and research is revealing new sources of MSC [27]. Placenta and umbilical cord recently emerged as an important source of stem cells since these neonatal tissues are readily available, avoiding the invasive harvesting procedures and ethical problems [28].

Overall, we demonstrated that even though different tissue sources are used, the characteristic morphology, markers and functional abilities of isolated cells converge towards the same pattern of mesenchymal stem cells. When we talk about the heterogeneity of isolated cells, there is a great debate regarding which of the tissular sources and isolation method can give rise to a more pure population, given the fact that we were able to identify at least two cellular types in our explants-isolated samples – mesenchymal and epithelial.

The *in vitro* aspect of the culture cells isolated from placenta and umbilical cord fulfilled the generally accepted criteria for MSCs, to adhere to plastic culture surfaces, to have fibroblast-like morphology and form spindles when reaching confluence (data not shown). When we analyzed the surface markers expression, we found positive expression for CD90, CD73, CD105, CD44 and CD29 (data not shown), and negative for CD34, CD45, and HLA-DR, consistent with mesenchymal stem cells populations and other reports on placenta and umbilical cord MSCs [4, 23, 25, 27, 28]. The low expression of CD105 detected by flow cytometry can be explained by the use of enzymatic digestion during the staining procedure, but its presence was strongly evidenced by immunocytochemical assessment. An important issue is the stability of MSCs markers in culture. Despite the loss of certain markers

following passaging [29, 30] and the gain of others [31], MSCs cultures retain their multipotential, indicating that these markers are unlikely to be reflective of the MSC's true 'stem cell' nature or its multipotentiality. Probably, many markers present on MSCs *in vivo* may be induced by microenvironment or be reflective of some of their functions, while being lost upon plastic adherence and exposure to culture media. At this moment, it appears that heterogeneity of MSCs, first noted by Friedenstein *et al.* [32] cannot be explained based on known surface markers alone. However, when a more detailed morphological and functional analysis was performed on PL- and UC-derived cells, we identified structures for intercellular junctions–desmosomes, which can be responsible of impaired function of UC-derived cells.

PL- and UC-isolated cells manifested a good proliferation potential as evidenced by Ki67 expression, which is a nuclear protein associated with cellular proliferation, being expressed in S, G1, G2 and M cellular cycle phases but not in G0 [33]. Vimentin is frequently used as marker of epithelial–mesenchymal transition, and is speculated that this molecule is involved in protein transport between nucleus and cytoplasmic membrane. Vimentin is well represented in control MSCs, but also in PL- and UC-derived cells.

Plastic abilities, meaning trilineage differentiation potential, as other characteristic of stem cells, was assessed for both PL- and UC-derived cells. Explants-isolated cells showed different differentiation degrees, depending on cell lineage they were induced into. However, UC-derived cells are to be preferred when considering "biological waste" samples in generation of cells for further use in regenerative medicine, due to their increased ability to differentiate towards adipogenic and osteogenic lineages.

To our knowledge, this is the first attempt to identify the adhesion behavior of PL- and UC-derived cells using a dynamic shear stress-generating system. For both VCAM and ICAM substrates, PL- and UC-derived cells presented decreased adhesion compared to control MSCs, as the shear stress progressively increases. This may be related to gestational age, isolation method, and also to the fact that adhesion proprieties are different for cells *in vivo* and *in vitro*.

⊟ Conclusions

Our research revealed that placenta and umbilical cord, considered "biological waste", can serve as a valuable source of mesenchymal stem cells (MSCs), but in the attempt to isolate stem cells, we should carefully look to the morphology, characteristic markers, and more important, function of cells we want to identify. Explants-isolated placenta and umbilical cord-derived cells represent a heterogeneous population, and additional purification methods are necessary in order to stimulate the use of these tissues in MSCs research or clinical applications.

Conflict of interests

The authors declare that they have no conflict of interests.

Acknowledgments

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