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# LNGFR (CD271) as Marker to Identify Mesenchymal Stem Cells from Different Human Sources: Umbilical Cord Blood, Wharton's Jelly and Bone Marrow

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## Abstract

Bone marrow has been considered the main source for isolation of multipotent mesenchymal stem cells for therapeutic approaches. However, alternative sources of mesenchymal stem cells have been sought because their frequency and differentiating capacity decrease with age and because they are not easy to obtain from healthy donors. These cells have been also described both in umbilical cord blood and in Wharton's jelly. To date, several studies have shown that CD271 is the most specific marker expressed of bone marrow mesenchymal stem cells before being cultured. The aim of this study was to determine whether it is possible to identify not cultured mesenchymal stem cells umbilical cord blood and Wharton's Jelly using this marker, as well as to compare the results found to those obtained in bone marrow.

Bone marrow, umbilical cord blood and Wharton's Jelly cells suspension were analyzed by flow cytometry in order to identify the $CD271^+/CD45^-$ population. Furthermore, we evaluated the potential to obtain mesenchymal stem cells from each source. Our results showed $CD271^+/CD45^-$ cells in bone marrow samples, although this population did not appeared in umbilical cord blood nor from Wharton's Jelly. However, the absence of this phenotype before culture samples had no relation with the ability to culture mesenchymal stem cells, specially taking into account that is was possible to obtain them from Wharton's Jelly but not from umbilical cord blood. Our findings suggest that CD271 is not an adequate marker to identify mesenchymal stem cells from human umbilical cord before culture.

**Keywords:** Bone marrow; CD271; Mesenchymal stem cells; Umbilical cord blood; Wharton's Jelly

## Introduction

Stem cell therapy has undergone a great development in recent years. Mesenchymal stem cells (MSCs) are widely characterized as undifferentiated cells with a high self-renew capacity [1]. Moreover, these cells are able to differentiate into multiple mesodermal tissues such as bone, cartilage, muscle, tendon or fat [2]. Hence, MSCs can be considered as a very promising tool for regenerative medicine.

Bone marrow (BM) has been the main source for isolation of multipotent MSCs. Because of their characteristics, BM-MSCs offer a great therapeutic potential which, in fact, has already been applied to develop cell based therapies to treat a wide range of pathologies [3]. Since the frequency and differentiating capacity of BM-MSCs decrease with age [4] and they are not easily obtain from healthy donors, alternative sources of MSCs are desirable.

Adipose tissue is an alternative source to obtain MSCs. These cells can be isolated from liposuctions in large numbers and grown easily under standard tissue culture conditions maintaining the multilineage differentiation capacity [5]. Moreover, MSCs can be also isolated from placenta [6] and synoviun [7] among other tissues.

The human umbilical cord (UC), a structure that connects the developing fetus to the placenta, could provide a new source of MSCs for clinical use. It contains two arteries and one vein, which are surrounded by mucoid connective tissues called Wharton's jelly (WJ). The UC possesses several desirable characteristics such as wide availability, a non-invasive and painless collection procedure and absence of ethical controversy [3]. MSCs have been described both in umbilical cord blood (UCB) and in WJ. Beside, instead of the typical blood cells, UCB was found to contain different populations of stem cells, a unique feature not shared with peripheral blood. Researchers have characterized the following stem cell populations from UCB: hematopoietic stem cells, multipotent non-hematopoietic stem cells and MSCs [2]. UCB derived MSCs show high morphological and molecular similarities to bone marrow derived MSC including the lack of hematopoietic surface antigens [8]. Furthermore, several authors have differentiated UCB-MSCs *in vitro* to osteogenic, chondrogenic, neural and hepatic lineages successfully [9-11]. On the other hand, the isolation of MSCs from UCB has been shown to be very difficult because of their low number, compared to BM. In this sense, little success has been reported in the literature regarding to the isolation, characterization and differentiation of MSCs from UCB. Several studies reported that MSCs can be isolated from only 20-63% of the cord blood units [1,12] questioning the feasibility of MSCs isolation and cultured from UCB [13].

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WJ was first observed by Thomas Wharton in 1656. This gelatinous substance is composed proteoglycans and various isoforms of collagen. The main role of the WJ is to prevent the compression, torsion and bending of the umbilical vessels which provide the bi-directional flow of oxygen, glucose and amino acids to a developing fetus, while also depleting the fetus and placenta of carbon dioxide and other waste products [14]. Human WJ-MSCs exhibit a high degree of self-renewal capacity and multi-lineage differentiation potential, similar to that of MSCs derived from BM [1].

Since their first description in BM [15], MSCs have been routinely isolated on the basis of their capacity to adhere to plastic tissue culture treated plates. However, this isolation strategy has the risks of haematopoietic cell contamination and heterogeneity. Giving this data, various attempts have been made to develop effective strategies to purify these cells directly from harvested BM [16]. *In vivo*, BM-MSCs phenotype has been difficult to establish because of their low frequency (0.001-0.01%) [2]. To date, the exact phenotype of BM-MSCs before culture remains unknown but several studies have shown that the low-affinity nerve growth factor receptor (LNGFR), now referred to as CD271, is the most specifically expressed marker by these cells [17]. Although CD271 was initially believed to be expressed by cells of the nervous system and plays a key role in the development, survival and differentiation of neural cells, it is now known to be a member of the low-affinity neurotrophin receptor and tumor necrosis factor receptor superfamily [18]. Thus, CD271 has been proposed as a versatile marker to selectively isolate and expand multipotent MSCs with both immunosuppressive and lymphohematopoietic engraftment-promoting properties [19-21].

Taking into account, the arising interest of MSCs for clinical use in different pathologies, huge efforts are being performed to optimize the harvest and purification of these cells from diverse sources. The aim of this work was to determine whether CD271 could be a useful marker to identify MSCs from UCB and WJ before culture.

## Materials and Methods

### Processing of bone marrow and culture of MSCs

BM aspirates were obtained from 23 donors by iliac crest aspiration under topical anaesthesia (Mepivacaine 2%, B. Braun, Melsungen, Germany) using a heparin-rinsed trocar and syringe (Mayne heparin 25000 IU, Mayne Pharma Espane S.L., Madrid, Spain). The BM aspirates were received in accordance with the ethical standards of the local ethical committee and all patients signed a detailed informed consent form prior to any intervention. Mononuclear cells (MNCs) were isolated from the BM aspirate on a Ficoll density gradient (Biocoll separating solution, 1.077 g/ml, Biochrom AG, Berlin, Germany) by centrifuging for 25 minutes at 400 g, 20°C without break. After washing twice in normal saline (Baxter International Inc, IL, United States), the cells were resuspended in culture medium consisting of Dulbecco´s modified Eagle´s medium (DMEM; Gibco Invitrogen, Paisley, United Kingdom) supplemented with 10% fetal bovine serum (FBS; Gibco Invitrogen), 100 U/ml penicillin (Gibco Invitrogen), and 100 µg/ml streptomycin (Gibco Invitrogen) and counted. This same medium will be used too for culturing the MSC derived from UCB and WJ. Cells ($10\times10^6$) were seeded into 25 $cm^2$ polystyrene flasks (Cultek, Madrid, Spain) and were cultured in a controlled atmosphere (37°C, 5% $CO_2$). After 48 h, the medium was first replaced and unattached cells removed. Thereafter, the medium was replenished every 2-3 days, and, when they were confluent, the cells were digested with trypsin-Ethylenediaminetetraacetic acid (EDTA)(Lonza, Basel, Switzerland) and passaged. BM-MSCs were used after 4-8 passages.

### Processing of umbilical cord blood and culture of MSCs

UCB was obtained from full-term deliveries (n=20) with the approved informed consent of the mothers and the collection was performed in accordance with the ethical standards of the local ethical committee. UCBs were collected by a standardized procedure using syringes that contained citrate dextrose and were processed within 24 hours after collection.

The isolation of MNCs was performed by a separation on a Ficoll (Biochrom) density gradient for 25 minutes at 400 g and 20°C without break. After washing twice in normal saline (Baxter), the cells were resuspended in culture medium and counted. Cells $10\times10^6$ were seeded into 25 $cm^2$ polystyrene flasks (Cultek) and cultured in a controlled atmosphere (37°C, 5% $CO_2$). After 48 h, the medium was first replaced and unattached cells removed. Thereafter, the medium was replenished every 2-3 days, and, when they were confluent, cells were digested with trypsin-EDTA (Lonza) and passaged.

### Processing of umbilical cord wharton's jelly and culture of MSCs

UC (n=10) were obtained after normal deliveries with the consent of the mother. Samples were collected into tubes containing RPMI medium (Gibco Invitrogen) supplemented with antibiotics. Each cord was rinsed with normal saline (Baxter) to remove as much blood as possible. The cord was cut in several pieces using a sterile scalpel. The WJ was carefully separated from the blood vessels and cut into small fragments with scissors and scalpels. Collagenase I (2 mg/ml; Sigma-Aldrich, St Louise, MO, United States) in DMEM (Gibco Invitrogen) was added to the fragments and the suspension was shaken and digested for 2-3 hours at 37°C. After digestion, the mixture was centrifuged at 500 g for 10 minutes and then digested with trypsin-EDTA (Lonza) for 30 minutes at 37°C with agitation. Afterwards, digestion was stopped by adding FBS. After filtering through a 40 µm cell strainer (BD Bioscience, San Jose, CA, USA) and centrifuging at 500 g for 10 min, cells were resuspended in culture medium and counted. Cells ($2.5\times10^6$) were seeded into 25 $cm^2$ polystyrene flasks (Cultek) were cultured in a controlled atmosphere (37°C, 5% $CO_2$). After 48 h, the medium was first replaced and unattached cells removed. Thereafter, the medium was replenished every 2-3 days, and, when they were confluent, the cells were digested with trypsin-EDTA (Lonza) and passaged.

### Flow cytometry analysis of MSCs from BM, UCB and WJ before culture

For phenotypic analysis of MSCs, $2.5\times10^6$ cells from each source were collected to identify the $CD271^+/CD45^-$ population. Cell surface staining for CD271 and CD45 expressions was performed by direct immunofluorescence. Cell suspension was washed in 1x Phosphate Buffer Saline (PBS; PAA Laboratories GmbH, Cölbe, Germany) and incubated with 20 µl FcR of blocking reagent (eBioscience, CA, United States) for 20 minutes at 4°C to avoid unspecific antibody binding. Antibodies against human CD271-PE (Miltenyi Biotec, Bergisch Gladbach, Germany) and human CD45-PE-Cy7 (eBioscience) were then added followed by incubation for 30 minutes at 4°C. As isotype controls, we used IgG1-PE (Miltenyi Biotec) and IgG1-PE-Cy7 (eBioscience). Fluorescence minus one (FMO) controls was stained in parallel with sequential omission of one antibody. In addition, another control was performed in which CD271 antibody was replaced by its isotype control rather than simply omitted. Once labelled, the cells

were resuspended in 0.5 ml 1x PBS (PAA Laboratories) and sieved using a 35 µm nylon filter (Cell Strainer, Becton Dickinson, NJ, USA). Dead cells were excluded by staining with 7-amino-actinomycin D (BD Pharmingen, San Jose, CA, United States). Samples were analyzed using a Becton Dickinson FACSAria with FACSDiva 6.1.2 software (Becton Dickinson).

### Inmunophenotyping of cultured MSCs isolated from BM, UCB and WJ

The antibodies used were directed against the surface marker panel proposed by The Mesenchymal and Tissue Stem Cell Committee of the ISCT [22]. Cell surface antigen phenotyping was performed on cultured MSCs at passages 4-8.

The MSCs phenotype was identified using antibodies against CD34-PerCpCy5.5 (BD Bioscience), CD73-PE (BD Pharmingen), CD90-FITC (eBioscience), CD105-APC (eBioscience) and CD45-PECy7 (eBioscience). Upon confluence, cells from one 75 $cm^2$ flask were removed by trypsin-EDTA and washed with 1x PBS (PAA Laboratories). The cells were then incubated with FcR blocking reagent (eBioscience) for 20 minutes at 4°C to avoid unspecific antibody binding. Next, cells were stained with the antibodies and their isotype controls for 30 minutes at 4°C. FMO controls were stained in parallel using the panel of antibodies with sequential omission of one antibody. After washing, the cells were resuspended in 0.5 ml 1x PBS (PAA Laboratories) and sieved using a 35 µm nylon filter cell strainer (Becton Dickinson). Samples were analyzed using a Becton Dickinson FACSAria with FACSDiva 6.1.2 software (Becton Dickinson).

### *In vitro* differentiation of MSCs from BM and WJ

Adipogenic differentiation was induced using NH AdipoDiff Medium (Miltenyi Biotec). In brief, cultured MSCs, isolated from each tissue, were resuspended in a final concentration of $5\times10^4$ cells/ml in NH AdipoDiff medium. The cells were seeded on 35 mm cell culture plates (BD Bioscience) and the medium replaced with fresh medium every 3rd day. After 2-3 weeks, large vacuoles started to appear. On day 21, adipogenic differentiation was assessed by Oil Red-O staining (Sigma-Aldrich).

Osteogenic differentiation was induced using NH OsteoDiff Medium (Miltenyi Biotec). Cultured MSCs, isolated from each tissue, were resuspended in final concentration of $5\times10^4$ cells/ml in NH OsteoDiff medium and seeded onto 35 mm cell culture plates (BD Bioscience). The medium was replenished every 3rd day. On day 10, differentiated cell cultures were stained with Sigma Fast Bcip/Nbt substrate (Sigma-Aldrich).

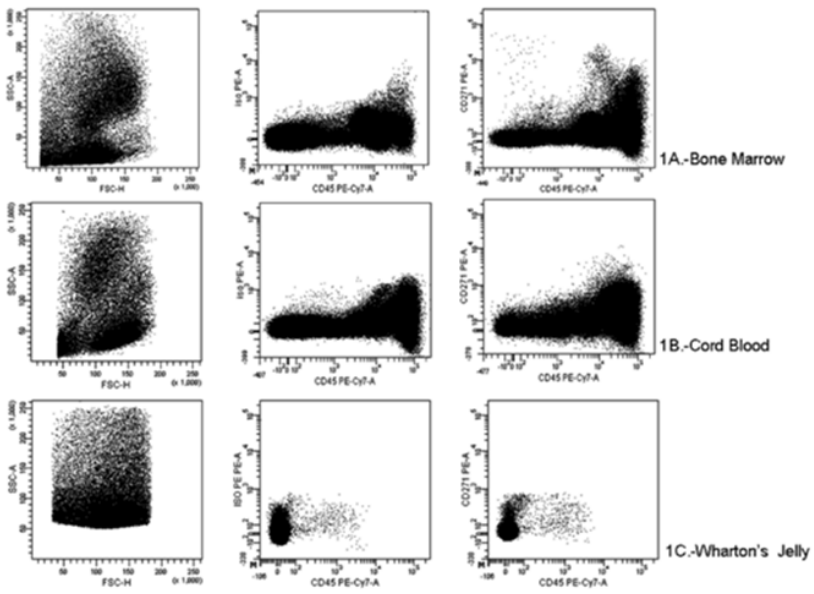
## Results

### Flow cytometry analysis of MSCs before culture

BM, UCB and cell suspension from WJ were analyzed in order to identify the $CD271^+/CD45^-$ population. The data obtained from BM have been published recently by our group [23]. In addition to this data, more samples showing similar low proportions and high variability of cells with MSCs phenotype among patients have been added. The new percentage of cells that presented the $CD271^+/CD45^-$ phenotype ranged from 0,0010% to 0,0201% and the mean percentage was 0.0042 ± 0.0010 (Mean ± typical error). In Figure 1(a) the identification of $CD271^+/CD45^-$ population in a BM sample is shown. Nevertheless, population with $CD271^+/CD45^-$ phenotype was not detected in neither UCB (Figure 1b) and WJ (Figure 1c) analyzed samples.

### Characteristics of cultured MSCs

Samples from BM, UCB and WJ were expanded *in vitro* to characterize the MSCs according to the criteria described by The Mesenchymal and Tissue Stem Cell Committee of the ISCT [24]. Under standard culture conditions, all MSCs cultures from BM and WJ adhered to treat plastic maintaining their fibroblast-like morphology,




Flow cytometry analysis for cells stained with CD271PE and CD45PECy7 monoclonal antibodies, and 7AAD for excluding dead cells and debris, are shown. Analysis was performed in (A) bone marrow, (B) cord blood and (C) Wharton`s jelly representative samples. Dot-plots FSC vs. SSC, CD45 vs. isotope control PE and CD45 vs. CD271 were used for identifying $CD271^+/CD45^-$ population after subtracting dead cells, debris and doublets. 500.000 events per dot-plot are shown.

**Figure 1:** Identification of $CD271^+/CD45^-$ population in fresh-isolated bone marrow, cord blood and Wharton`s Jelly samples.

as originally described by Friedenstein et al. [15]. Nevertheless, it was observed that cultured cells from UCB did not present this criterion. These cultured cells appeared as a heterogeneous adherent cell population, combining the spindle-shaped and the round-shaped appearance. The morphology of cells obtained after culture from each source is shown in Figure 2.

Passage 4-8 MSCs were assessed for their potential to differentiate into adipogenic and osteogenic lineages. All expanded MSCs from BM and WJ were able to differentiate into these 2 lineages as revealed by labelling with specific stains. Adipogenesis was indicated by the deposition of neutral lipid vacuoles that stained with Oil Red-O; osteogenesis, by increased alkaline phosphatase-positive cells (data not shown). In the case of UCB, cultured cells could not be successfully passaged and no adequate number of MSCs was obtained in order to test the differentiation capacity.

Immunological characterization of cultured MSCs (passages 4-8) by flow cytometry were positive for the markers CD73, CD90 and CD105 and negative for the hematopoietic markers CD34 and CD45. As it is shown in Figure 3(a), cultured MSCs (passage 6) from BM present $CD34^-CD45^-CD90^+CD73^+CD105^+$ phenotype. In all passages analyzed (4-8) from BM-MSCs showed the similar percentage of cells with this phenotype. Figure 3(b) shows the same analysis as BM MSCs in cultured MSCs from WJ. However, the percentage of cells with phenotype $CD34^-CD45^-CD90^+CD73^+CD105^+$ was lower in WJ-MSCs (63.6%) than in BM-MSCs (95.2%). This difference, as it is shown in dot-plots 3.B CD105 vs CD90 and CD105 vs. CD73, was due to the fact that CD105 expression was clearly lower in WJ-MSCs than BM-MSCs. Figure 3(b) represents MSCs in passage 6, meanwhile analysis done in passage 4 showed 25% and passage 8 showed 72% of cells with phenotype $CD34^-CD45^-CD90^+CD73^+CD105^+$ (data not shown). Moreover, similar data has been found in flow cytometry analysis of cultured MSCs from adipose tissue (data not shown). As it has been indicated above, it has not been able to obtain adequate number of MSCs from UCB to carry out immunophenotyping analysis of these cells. The obtained results are summarised in Table 1.

## Discussion

BM-MSCs are the most intensely studied progenitor/stem cell types in cell therapy. Clinically, aspirating BM from patient is an invasive and

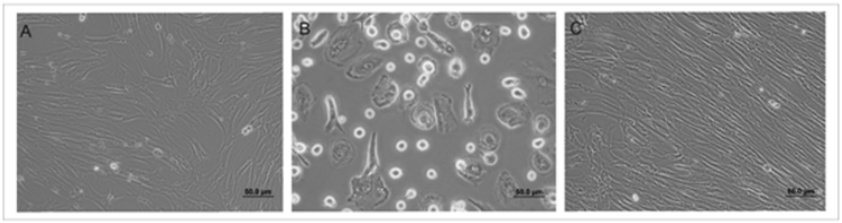
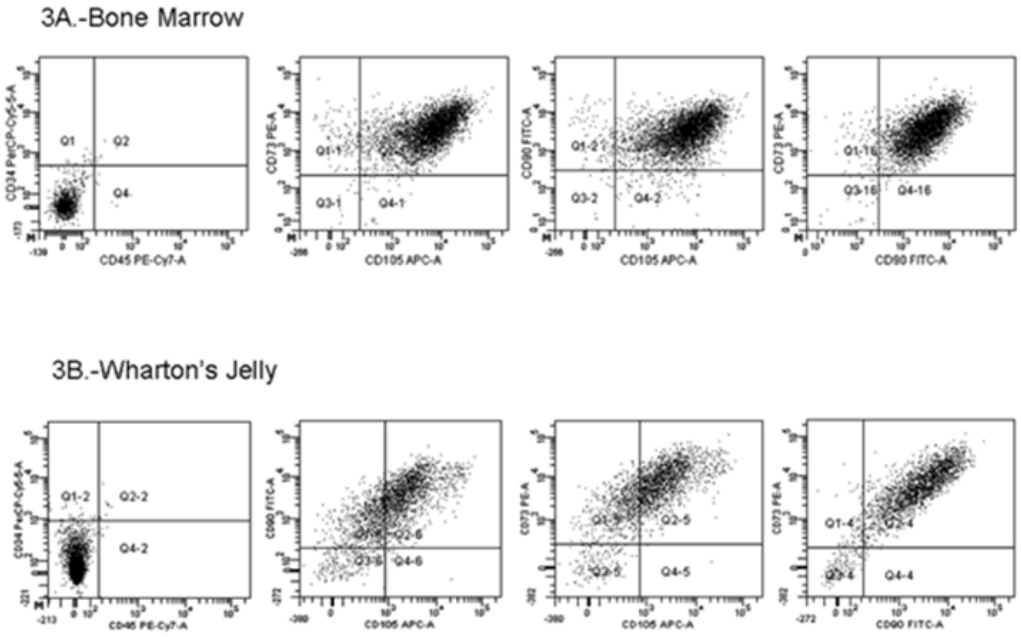



**Figure 2:** Microscope appearance of MSCs in passage 1. A) BM-derived MSCs; B) UCB-cells; C) WJ-derived MSCs.




Flow cytometry analysis for cells stained with CD90FITC, CD73PE, CD34PerCpCy5.5, CD105APC and CD45PECy7 monoclonal antibodies are shown. Analysis was performed in (A) bone marrow and (B) Wharton`s jelly representative cultures. Dot-plots CD45 vs. CD34, CD105 vs. CD73, CD105 vs. CD90 and CD90 vs. CD73 were used for characterizing $CD34^-CD45^-CD73^+CD90^+CD105^+$ population after subtracting dead cells, debris and doublets. 50.000 events per dot-plot are shown.

**Figure 3:** Phenotypic characterization of cultured MSCs from bone marrow and Wharton`s Jelly samples.

**Table 1:** Summarized MSCs characteristics from different sources analyzed before and after culture.

| | Flow cytometry before cultured **$CD271^+/CD45^-$** | Cultured MSC Phenotype | Flow cytometry analysis Cultured MSC (Passage6) **$CD34^-CD45^-CD90^+CD73^+CD105^+$** | *In vitro* differentiation Adipogenesis Osteogenesis |
|---|---|---|---|---|
| **BM** | 0.0042% ± 0.001 | Fibroblast-like | 95.2% | Yes |
| **UCB** | 0% | Heterogeneous population | - | - |
| **WJ** | 0% | Fibroblast-like | 63.6% | Yes |

painful process so alternative sources of MSCs are required to apply these cells in regenerative medicine. Because of its availability, non-invasiveness and potentiality, UC is a very interesting source of MSCs for this purpose.

In this study, we investigated the use of CD271 as a marker to identify MSCs from UC, both from UCB as well as from WJ, before culture. $CD271^+/CD45^-$ phenotype has been published as an adequate marker to identify MSCs from BM and lipoaspirates before culture [19,21]. However, we have not found this phenotype in the mononuclear fraction from UCB. Similar results have been recently published by Watson et al. (2013), in spyte of using a different method to identify CD271+ cells from UCB (antibody conjugated-microbeads) [24]. One explanation to this could be that MSCs circulation in blood is much lower than that in BM [25]. Researchers have used a variety of methods for isolation, culture and characterization of MSCs from UCB with controversial results [26]. The ability to obtain MSCs from UCB seems to be restricted in comparison with the potential of BM under similar culture conditions [27].

We also accomplished phenotype characterization of the cells from WJ and the results of the cytometric analysis did not show a population with this phenotype; conversely, it was possible to culture MSCs from WJ *in vitro*. Although CD271 has been described as the most selective marker for the characterization and purification of human BM-MSCs [28], this marker could be not so selective for MSCs from WJ before culture. Our results are in concordance with other authors whose data show the absence of CD271 cells after enzymatic digestion of WJ [3] and after isolation of cells by explants methods [29].

We tried to culture MSCs from UCB using the technique already established in our laboratory for BM. No expansion of adherent cells with MSCs phenotypic characteristics was obtained. We observed heterogeneous population of adherent cells, round-shaped and spindle-shaped appearance (Figure 2). Our results are in concordance with Perdikogianni et al. [30], who were not able to obtain MSCs from UCB by using standard methods of culture. These researchers suggest this could be related the low proportion of MSCs present in UCB. In addition, according to the literature, even in the studies that report successful growth of MSC-like cells from UCB these cells were not found in all the UCB samples analyzed [27,31]. The percentage of UCB samples generating MSCs according to different reports ranges from 23% to 63% [1,8]. In the present study, 20 samples of UCB were processed to obtain MSCs. The results were no adherent MSCs cells although a heterogeneous population of cells where stand out big round cells and spindle-shaped cells in a low proportion was observed. Studies have shown that UC-MSCs have the potential of *in vivo* and *in vitro* differentiation into many cell types. However, up until now, the question of which is the optimal method for efficiently isolating MSCs from UC still remains unanswered [32]. In concordance to Perdikogianni et al. [30], due to the differences observed concerning the success rate of isolating MSCs from UCB compared with 100% success rate for BM-MSCs, the use of UCB as a source of MSCs should be questioned for clinical use.

In contrast, WJ has been shown to be a suitable source of MSCs with practical advantages [33]. Several studies demonstrated that cells derived from WJ have similar characteristics to BM-MSCs [1,34]. Despite the absence of CD271 cells in samples of WJ before culture, in all WJ processed in our study we obtained adherent cells with fibroblast-like morphology and potential to differentiate into adipogenic and osteogenic lineages. The phenotypic characterization of cultured WJ-MSCs showed that MSCs were positive for CD73, CD90 and CD105 markers as well as negative for CD34 and CD45. Cultured BM-MSCs are homogeneously and strongly positive for CD73, CD90 and CD105. However, CD105 and CD73 are also expressed on skin fibroblast [35], cells with a much lower ability to proliferate and differentiate, compared to MSCs [23]. This implies that sole demonstration of CD105 and CD73 expression without CD90 on adherent cultures cells is insufficient to prove their MSCs identity [36]. Our results showed that the percentage of cells with phenotype $CD34^-$ $CD45^-$ $CD73^+$ $CD90^+$ $CD105^+$ was lower in WJ-MSCs (63,6%) than in BM-MSCs (95, 2%). However, this percentage shows a variation with the passage of the cells because of the increase of the percentage of positive cells for CD105 marker. Moreover, similar data in immunophenotyping analysis of culture MSCs from adipose tissue (data not shown) has been found.

In summary, our results showed $CD271^+/CD45^-$ cells in bone marrow samples although this population did not appear in samples from UCB and WJ. However, the absence of this phenotype in fresh samples has no relation with the ability to culture MSCs, considering that these cells were found in WJ samples but not in UCB samples. Therefore, in spite of $CD271^+/CD45^-$ phenotype has been accepted as a good marker to identify of MSCs from BM and lipoaspirates samples before culture, our findings suggest that CD271 is not an adequate marker for identification of MSCs before culture from human UC.

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