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Multipotent Stem Cells from Umbilical Cord: Cord Is Richer than Blood!

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ABSTRACT

The identification of mesenchymal stem cell (MSC) sources that are easily obtainable is of utmost importance. Several studies have shown that MSCs could be isolated from umbilical cord (UC) units. However, the presence of MSCs in umbilical cord blood (UCB) is controversial. A possible explanation for the low efficiency of MSCs from UCB is the use of different culture conditions by independent studies. Here, we compared the efficiency in obtaining MSCs from unrelated paired UCB and UC samples harvested from the same donors. Samples were processed simultaneously, under the same culture conditions. Although MSCs from blood were obtained from only 1 of the 10 samples, we were able to isolate large amounts of multipotent MSCs from all UC samples, which were able to originate different cell lineages. Since the routine procedure in UC banks has been to store the blood and discard other tissues, such as the cord and/or placenta, we believe our results are of immediate clinical value. Furthermore, the possibility of originating different cell lines from the UC of neonates born with genetic defects may provide new cellular research models for understanding human malformations and genetic disorders, as well as the possibility of testing the effects of different therapeutic drugs. STEM CELLS *2008;26: 146–150*

Disclosure of potential conflicts of interest is found at the end of this article.

INTRODUCTION

Mesenchymal stem cells (MSCs) are undifferentiated cells that are able to self-renew and that have a high proliferative capacity. These cells comprise a rare population of multipotent precursors that are capable of supporting hematopoiesis. Moreover, several reports suggest that MSCs were able to differentiate into various cell types, including chondrocytes, osteocytes, adipocytes, myocytes, and neurons [1–4]. MSCs can be isolated from different tissues, such as bone marrow (BM), adipose tissue, dental pulp, placenta, and umbilical cord blood (UCB), and from a variety of fetal tissues, such as spleen, lung, pancreas, kidneys, and amniotic fluid during midgestation [5–10]. Phenotypic and genetic evidence suggests that MSCs are an immature cell type, being a potentially useful model for developmental biology studies in normal and disease background, in addition to their therapeutic potential.

BM has been considered as one of the main sources of MSCs for both experimental and clinical applications, and most of the knowledge concerning MSCs comes from BM studies. However, MSCs from BM decrease significantly with age [11, 12], and their isolation is invasive and can cause infection, bleeding, and chronic pain. In past decades, human UCB has been regarded as an alternative source to BM cell transplantation and therapy because of its hematopoietic and mesenchymal components. Human UCB is obtained after full-term delivery of the newborn from a sample that would inevitably be discarded. The process is noninvasive, painless, and without harm for the mother or the infant.

Hematopoietic stem cells (HSCs) from UCB have already been proven to be useful in treating various hematological disorders [13–16]. However, the presence of MSCs in UCB is controversial. Some researchers succeeded in isolating these cells [8, 17–19], whereas others failed or obtained a low yield [20–22]. More recently, some groups have reported success in isolating and establishing MSCs cultures from umbilical cord (UC) vein and UC stroma, also called Wharton's jelly [3, 20, 23–25]. According to Tondreau et al., the discrepant results in isolating MSC from UCB might be explained by different methodologies used for obtaining and culture these cells [26]. To address this issue, we compared 10 samples of UCB and UC, obtained from the same donors, regarding the presence of MSCs, as well as the differentiation potential in culture. Our results show that UC are rich in MSCs that are able to differentiate into various cell lines in vitro, whereas MSCs from blood were obtained from only 1 of the 10 samples. Based on these findings, we strongly suggest storing UC, in addition to UCB, for future therapeutic applications and scientific investigation purposes.

MATERIALS AND METHODS

Harvesting of UCB and UC

Ten human UCB and UC matching units were collected after informed consent was obtained from the mother in accordance with the ethical committee of Institute of Bioscience of University of São Paulo and Jesus José e Maria Hospital. Informed consent was obtained from all subjects. All studies and laboratory procedures

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were carried out in the Human Genome Research Center, São Paulo, Brazil.

From each sample, UCB was harvested and conserved with 100 mM EDTA anticoagulant at 22°C. Sections of 8–10 cm of umbilical cords, routinely discarded, were internally washed with phosphate-buffered saline (PBS) containing 300 U/ml penicillin and 300 μg/ml streptomycin (Gibco, Grand Island, NY, http://www.invitrogen.com) and immediately immersed in Dulbecco's modified Eagle's medium-low glucose (DMEM-LG; Gibco) supplemented with 10% fetal bovine serum (FBS; Gibco), 300 U/ml penicillin, and 300 μg/ml streptomycin. All samples were processed within 12–15 hours after collection.

Isolation and Culture of Adherent Cells from UCB

After blood dilution of 3:1 with RPMI 1640 medium (Gibco), mononuclear cells (MNCs) were isolated by density gradient centrifugation at 400*g* for 30 minutes at room temperature using Ficoll-Paque Premium (GE Healthcare, Little Chalfont, U.K., http://www.gehealthcare.com). MNCs were washed twice with PBS and resuspended in proliferation medium consisting of DMEM-LG, 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin. Cells were plated at a density of 5×10^7 cells per ml in culture flasks (25 cm^2) and maintained at 37°C in a humidified atmosphere containing 5% CO_2. After 24 hours of incubation, nonadherent cells were removed, and culture medium was replaced every 3 days. Adherent cells were cultured until they reached 80%–90% confluence.

Isolation and Culture of Adherent Cells from UC

UCs were filled with 0.1% collagenase (Sigma-Aldrich, St. Louis, http://www.sigmaaldrich.com) in PBS and incubated at 37°C for 20 minutes. Each UC was washed with proliferation medium, and the detached cells were harvested after gentle massage of the UC. Cells were centrifuged at 300*g* for 10 minutes, resuspended in proliferation medium, and seeded in 25-cm^2 flasks at a density of 5×10^7 cells per ml. After 24 hours of incubation, nonadherent cells were removed and cultivated as described above.

Immunophenotyping

To analyze cell-surface expression of typical protein markers, adherent cells were incubated with the following anti-human primary antibodies: CD29-PECy5, CD34-PerCP, CD31-phycoerythrin (PE), CD45-fluorescein isothiocyanate (FITC), CD90-R-PE, CD117-PE, human leukocyte antigen (HLA)-ABC-FITC, HLA-DR-R-PE (Becton, Dickinson and Company, Franklin Lakes, NJ, http://www.bd.com), and SH3 (kindly provided by Dr. Kerkis, Instituto Butantã, São Paulo). Unconjugated markers were reacted with anti-mouse PE secondary antibody (Guava Technologies, Hayward, CA, http://www.guavatechnologies.com). A total of 10,000 labeled cells were analyzed using a Guava EasyCyte flow cytometer running Guava ExpressPlus software (Guava Technologies).

Cell Differentiation Procedures

To evaluate MSC properties, adherent cells (third passage, at 80%–90% confluence) were subjected to adipogenic, chondrogenic, myogenic, and osteogenic differentiation in vitro, according to established protocols [1, 3]. Normal human dermal fibroblasts were used as a negative control in the differentiation studies.

Adipogenic Differentiation

Subconfluent cells were cultured in proliferation medium supplemented with 1 μM dexamethasone (Sigma-Aldrich), 500 μM 3-isobutyl-1-methylxanthine (Sigma-Aldrich), 60 μM indomethacin (Sigma-Aldrich), and 5 μg/ml insulin (Sigma-Aldrich). Adipogenic differentiation was confirmed on day 21 by intracellular accumulation of lipid-rich vacuoles stainable with oil red O (Sigma-Aldrich). For the oil red O stain, cells were fixed with 4% paraformaldehyde for 30 minutes, washed, and stained with a working solution of 0.16% oil red O for 20 minutes.

Chondrogenic Differentiation

A pellet culture system was used for chondrogenesis. Cells (2.5×10^5) were centrifuged in a 15-ml polypropylene tube at 500*g* for 5 minutes, and the pellet was resuspended in 10 ml of basal medium consisting of DMEM-LG supplemented with 100 nM dexamethasone, 50 μM ascorbic acid-2 phosphate (Sigma-Aldrich), 1 mM sodium pyruvate (Gibco), and 1% ITS-Premix (Becton Dickinson). Without disturbing the pellet, cells were resuspended in 0.5 ml of chondrogenic differentiation medium consisting of basal medium supplemented with 10 ng/ml transforming growth factor-β1 (R&D Systems Inc., Minneapolis, http://www.rndsystems.com). On day 1, tubes were flipped gently to acquire a single floating cell sphere. Medium was changed every 3–4 days, and cells were fixed on day 21 with 4% paraformaldehyde. Cryosections (10 μm thick) were stained with toluidine blue to demonstrate extracellular matrix mucopolysaccharides.

For chondrogenic differentiation in monolayer culture, adherent cells were cultured in chondrogenic differentiation medium for 21 days. Chondrogenesis was demonstrated by staining with toluidine blue.

Osteogenic Differentiation

To promote osteogenic differentiation, subconfluent cells were treated with proliferation medium supplemented with 50 μM ascorbate-2 phosphate, 10 mM β-glycerophosphate (Sigma-Aldrich) and 0.1 μM dexamethasone for 21 days. Osteogenesis was demonstrated by accumulation of mineralized calcium phosphate assessed by von Kossa stain. Briefly, cells were stained with 1% silver nitrate (Sigma-Aldrich) for 45 minutes under ultraviolet light, followed by 3% sodium thiosulfate (Sigma-Aldrich) for 5 minutes, and then counterstained with van Gieson stain.

Myogenic Differentiation

For myogenic differentiation, adherent cells from UC were cultured in proliferation medium supplemented with 0.1 μM dexamethasone (Sigma-Aldrich), 50 μM hydrocortisone (Sigma-Aldrich), and 5% horse serum (Gibco) for 30 days. After that, cells were fixed with 4% paraformaldehyde and blocked with a blocking solution containing 10% fetal bovine serum, 5% bovine serum albumin, and 0.1% Triton X-100 in PBS for 1 hour. Primary antibody was added at a concentration of 1:100 for Myosin (M7523; Sigma-Aldrich) and 1:20 for dystrophin (VP-D508; Vector Laboratories) and incubated at room temperature for 2 hours. After several washes, cells were incubated with secondary antibodies against mouse IgG tagged to FITC (green) or rabbit IgG tagged to cyanine 3 (red) for 2 hours at room temperature. Immunostaining controls were done in the same conditions but lacked the primary antibody. Slides were counterstained with 4,6-diamidino-2-phenylindole and mounted in Vectashield (Vector Laboratories, Burlingame, CA, http://www.vectorlabs.com) solution. All images in the same set (samples and controls) were obtained using the same photographic parameters of exposition and speed. Images were captured using the Axiovision 3.0 image analysis system (Carl Zeiss, Jena, Germany, http://www.zeiss.com).

RESULTS

Isolation and Culture of Adherent Cells from UCB and UC

After plating MNCs from UCB, different cell types were observed. Most of them displayed an oval morphology (Fig. 1A), with few or no cytoplasmatic extensions. Some cells had an MSC-like phenotype (Fig. 1B), but most of them did not spread, migrate, or proliferate after 14 days in culture. Although we could isolate and expand MSCs from only one of the UCB units (data not shown), all the samples of UC generated primary adherent cultures, with cells displaying an MSC-like phenotype. After 4 days in culture, these cells grew in colonies, reaching confluence after 10–14 days. Most of the cells were spindle-

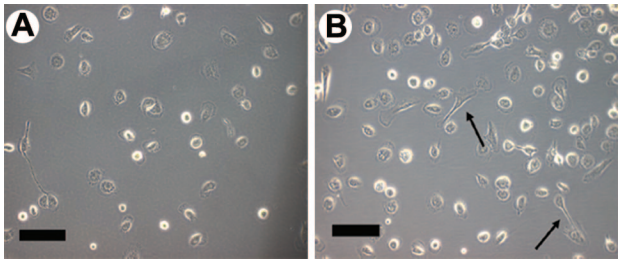

Figure 1. Morphology of adherent cells when isolated from umbilical cord blood. **(A):** Cells cultured for 4 days after initial plating displayed a round morphology. **(B):** Cells with an MSC-like phenotype (arrows) were also observed at day 4 but only in a few samples. However, these cells did not proliferate further than 14 days. Scale bars = 100 μm.

shaped, resembling fibroblasts. Some clusters of cells with endothelial appearance, which spread weakly and practically did not proliferate, could also be observed. After the second passage, adherent cells were constituted by homogeneous cell layers with an MSC-like phenotype (Fig. 2A–2D). The number of MSC from UC decreased slightly after freezing and thawing, and the remaining viable cells were successfully expanded on consecutive days (data not shown).

Immunophenotypic Analyses

All adherent cells derived from UC did not express hematopoietic lineage markers (CD34, CD45, and CD117) and endothelial markers (CD31), as assessed by flow cytometry. In addition, the majority of cells expressed high levels of adhesion markers (CD29 and CD90) and MSC markers (SH3). The isolated cells from UC were also positive for HLA-class I (HLA-ABC) but negative for HLA-class II (HLA-DR) (Fig. 3). In comparison with our fibroblast control, no obvious difference in the expression of these surface antigens could be observed (data not shown). Thus, the MSC property of isolated cells was further confirmed with cell differentiation studies.

Multilineage Differentiation Potential

The plasticity of adherent cells obtained from cord blood (CB) and UC was assessed 3 weeks after mesodermal induction. Osteogenic, adipogenic, and chondrogenic differentiation was demonstrated by the calcium deposits, lipid vacuoles, and mucopolysaccharide-rich extracellular matrix, respectively. No evident differences in MSCs from CB and UC differentiation potential were detected. Furthermore, an osteogenic, adipogenic, or chondrogenic phenotype was not observed in induced fibroblasts (negative controls; Fig. 4).

In addition, the potential of adherent cells from UC to differentiate into skeletal muscle cells was investigated. The myogenic differentiation was demonstrated by the expression of myogenic markers (myosin and dystrophin). As shown in Figure 5, myosin and dystrophin were specifically expressed in the differentiated cells rather than in untreated cells (noninduced controls). Together, these results confirmed the mesenchymal nature of the isolated cells and their multipotency.

DISCUSSION

In this study we compared, for the first time, the efficiency in obtaining MSCs from match-paired UCB and UC samples harvested from the same donors, which were processed simultaneously and under the same culture conditions. Although MSCs from blood were obtained from only 1 of the 10 samples, we were able to generate primary MSCs cultures from all cord

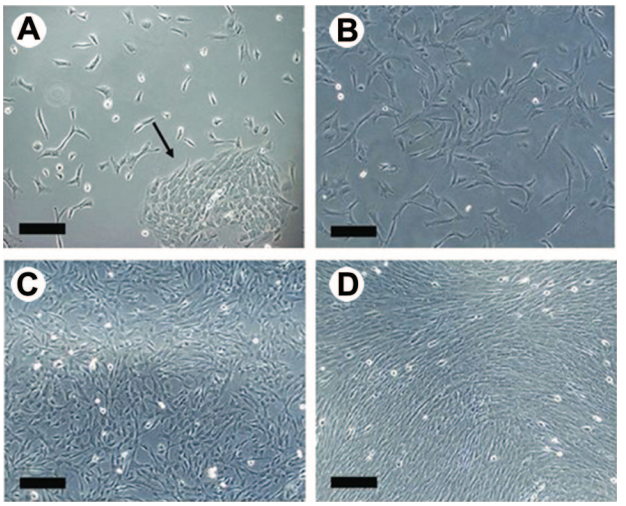

Figure 2. Adherent cells in primary cultures of UC. **(A–D):** The morphology and growth of cells with an MSC-like phenotype after 4, 7, 10, and 14 days of culturing, respectively. Some residual clusters of cells with endothelial appearance (arrow) could also be identified. Scale bars = 200 μm.

samples with a 100% yield. MSCs from UC are isolated by a fast and simple procedure using short enzymatic digestion (which provides a large number of cells without risk to the donor) and can easily be expanded in vitro, stored cryogenically, and thawed. MSCs have been reported to be isolated from UC by others, using different protocols [3, 23, 24, 27–30]. Likewise, difficulties in obtaining MSCs from UCB have been reported previously [8, 17]. Based on our experience, the efficiency in isolating MSC from blood in approximately 100 umbilical cord units stands around 10% (unpublished data).

Crucial parameters for the isolation of MSCs from UCB, such as time between collection and processing, the volume of samples, and the amount of MNC have been already described. Even so, the MSCs yield is never greater than 60% [19]. Recently, MSCs have successfully been derived from CD133+ hematopoietic stem cells originated from UCB [26]. However, this method would not allow the simultaneous isolation of hematopoietic and mesenchymal progenitors.

In mobilized peripheral blood, controversial results have been reported about the presence or absence of MSCs [22, 31–33]. da Silva Meirelles et al. reported that MSCs could not be detected in circulating blood in adult rats and suggested that all tissues have MSCs reservoirs localized in the perivascular niche [33]. According to these authors, it might be possible that during harvesting procedures only a few cells would detach from this perivascular niche as a result of needle syringe friction in blood vessel wall or the pressure applied in the UC during blood collection. On the other hand, a full success in obtaining MSCs from UC by our group and others might be explained by enzymatic dissociation processes within the UC perivascular niche. It is questionable whether the results observed in rats can be extrapolated to human samples and whether both MSC populations described here share the same genetic repertoire.

It has been suggested that the removal of myeloid and osteoclast-like cells seems to favors the isolation of MSCs from UCB [19]. Therefore, cocultivation with other hematopoietic cell types could inhibit the proliferation of the few MSCs present in the sample. Although some adherent primary cultures of UC contained cells with an endothelial phenotype, in our conditions, such contaminants did not interfere with MSCs growth. Others have also suggested that MSCs occurred at a low

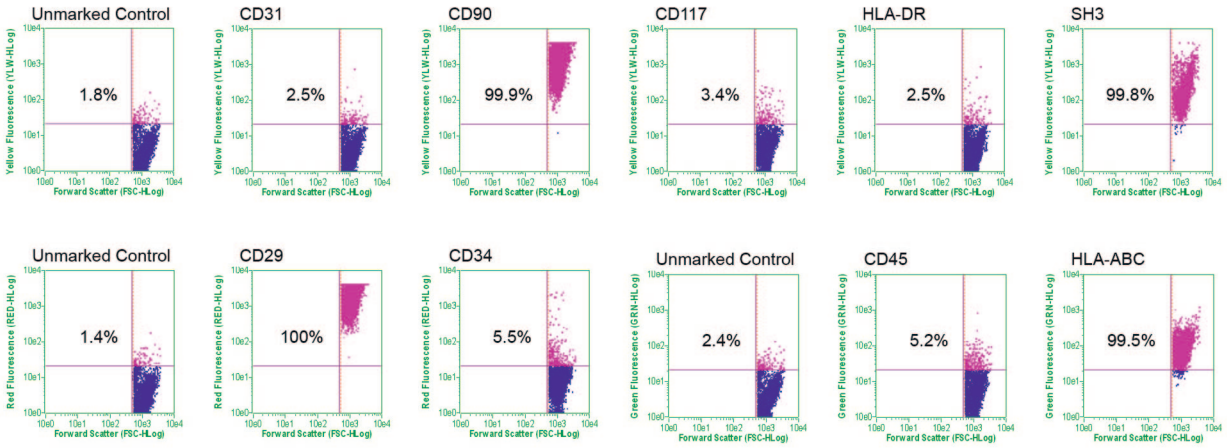

Figure 3. Immunophenotyping of adherent cells isolated from umbilical cord. Values represent the mean percentage of all assessed cells positively stained by the indicated antigens and analyzed by flow cytometry. Graphs show forward scatter versus fluorescence intensity. Abbreviations: HLA-ABC, human leukocyte antigen-ABC; HLA-DR, human leukocyte antigen-DR; SH3, Src homology 3.

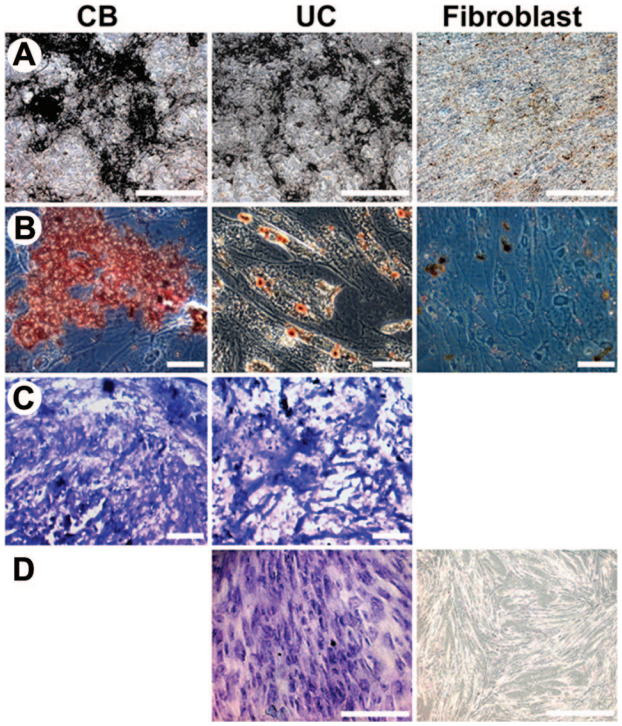

Figure 4. Differentiation potential of adherent cells isolated from CB and UC. **(A):** Osteogenic differentiation from adherent cells from CB and UC was demonstrated by calcium deposition shown by von Kossa stain. Scale bar = 50 μm. **(B):** Adipogenesis was detected by the formation of intracytoplasmic lipid droplets stained with oil red O. Scale bar = 200 μm. **(C):** Cell spheres from CB and UC were stained with toluidine blue to confirm chondrogenic differentiation. Mucopolysaccharide-rich extracellular matrix is shown in pinkish metachromatic areas. **(D):** Chondrogenic differentiation in monolayer culture of MSC from UC was demonstrated by stained with toluidine blue. Scale bars = 50 μm. **(A, B, D):** Osteogenic, adipogenic, or chondrogenic phenotype was not observed in induced fibroblasts (negative controls). Scale bars = 200 μm. More details are given in Materials and Methods. Abbreviations: CB, cord blood; UC, umbilical cord.

frequency in UCB and that their survival could be affected by different culture conditions [26]. However, our results show that the low yield of MSCs in UCB is likely not due to different culture methodologies.

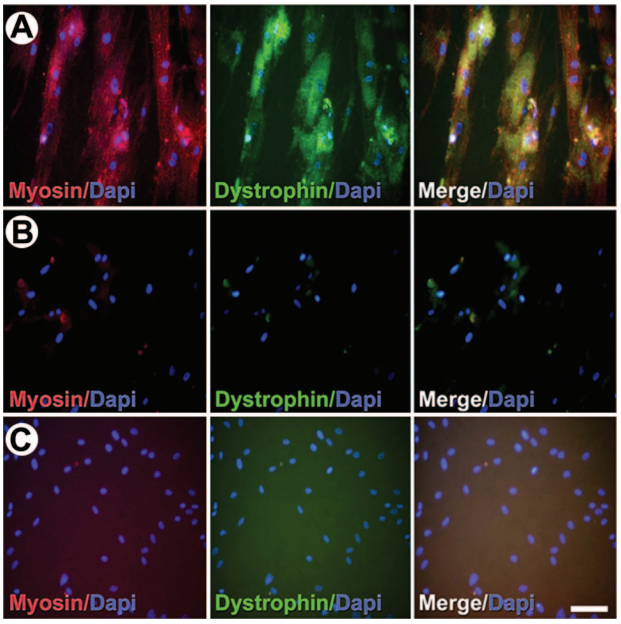

Figure 5. Myogenic differentiation potential of adherent cells isolated from umbilical cord. **(A):** Myogenic differentiation was assessed by immunocytochemistry. Induced cells were strongly labeled with anti-human myosin monoclonal antibody (Sigma-Aldrich) in red and, with anti-human dystrophin (dys1) monoclonal antibody (Vector Laboratories) in green. Counterstaining with Dapi (in blue) was used to identify all nuclei. **(B):** Noninduced controls. **(C):** Negative controls (without the first antibody). Scale bar = 50 μm. Abbreviation: Dapi, 4,6-diamidino-2-phenylindole.

An important issue in cellular therapy studies is the availability of alternative stem cell sources and the efficacy of isolation techniques to yield a reasonable amount of viable cells that could be successfully expanded. Despite the advantages of HSC from UCB in hematopoietic reconstitution [13–15], results from the present study demonstrated that UC, and not UCB, is the best choice for isolating MSCs for future applications. Until very recently, BM has been considered the main source of MSCs. Panepucci et al. demonstrated that MSCs derived from UC and BM are highly similar at the transcriptional level, reinforcing the usefulness of UC from neonates [34].

In short, based on the present results and other studies, we believe that UC is the easiest obtainable biological source of MSCs. Storing UCB in private or public banks has been recently the subject of many ethical dilemmas. Here we show that regardless of being public or private, these banks are discarding a precious source of MSCs. In addition, since human UC contain a significant amount of MSCs, we suggest donors to split samples, storing in both public and private banks. Storing both UCB and UC would allow maximum recovery of HSCs and MSCs for possible therapeutic applications in the future. Furthermore, the possibility of originating different UC-derived cell lines from babies born with human malformations and genetic disorders may provide new research models for understanding pathological mechanisms responsible for these conditions. In addition, storing both UCB and UC would also create the possibility of testing the effects of therapeutic drugs in these different cell lineages.

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DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

The authors indicate no potential conflicts of interest.

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