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A Rapid, Simple, and Reproducible Method for the Isolation of Mesenchymal Stromal Cells from Wharton's Jelly Without Enzymatic Treatment

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The co-infusion of mesenchymal stromal cells (MSCs) with hematopoietic stem cells could improve the hematopoietic engraftment after cord blood transplant. Adult bone marrow is the major source of MSCs for cell therapy. However, bone marrow aspiration involves an invasive procedure and, in the case of a cord blood transplant, requires the use of a third party. The umbilical cord matrix, called Wharton's jelly (WJ), was previously shown to be a valuable source of MSCs. However, the process of cell separation is not standardized and needs to be optimized. In this study, we focused on the efficiency of the isolation procedure and expansion of cells from WJ MSCs isolated from human full-term umbilical cords. MSCs were isolated from the WJ without enzyme digestion or dissection. The procedure was based only on the plastic adhesion capacities of MSCs. Briefly, umbilical cord segments of 5–10 cm were cut longitudinally and plated with the WJ onto a plastic surface for 5 days in an appropriate culture medium. After removing the cord segment, the culture was pursued until subconfluency. The number of cells and their phenotypes, clonogenic capacities, differentiation capacities, immunomodulation, and hematopoietic supportive functions were evaluated. Using this method, we were able to isolate MSCs from all human umbilical cords analyzed ($n = 50$). We obtained a mean of 1.4×10^8 cells at the second passage and $>7 \times 10^9$ cells at the third. The expanded cells expressed characteristic markers and presented typical functional properties of MSCs such as differentiation capacities, immunologic properties, and hematopoietic supportive functions. In conclusion, we have established a simple, rapid, and reproducible protocol to isolate abundant MSCs from short segments of umbilical cords.

Introduction

Umbilical cord blood is currently used as a transplantable source of hematopoietic stem cells. However, the volume of cord blood collected constitutes a limiting factor and, in most cases, only yields sufficient stem cells for a child recipient [1,2]. This problem could possibly be overcome through the cotransplantation of the umbilical cord blood with mesenchymal stromal cells (MSCs), which are known to support hematopoiesis and to provide niches for hematopoietic stem cells during engraftment [3–6].

Traditionally, adult bone marrow (BM) represents the most common source of MSCs for clinical applications [7]. MSCs could be isolated from various tissues, including adipose tissue, cord blood, dental pulp, and the conjunctive tissue of the umbilical cord called Wharton's jelly (WJ) [8]. Isolation of fibroblast-like cells from the WJ of the umbilical cord was originally described in 1991 [9]. Since their initial identification, a relatively limited number of studies have been conducted on these cells. Several groups have described that they fulfill the criteria of MSCs [10–16]. These cells can adhere to plastic, express characteristic MSC surface markers, and can differentiate into cells of mesodermal origin, including bone, cartilage, and adipose tissue [17].

Technical procedures to isolate MSCs from the WJ are poorly investigated and vary dramatically depending on the authors. Although the enzymatic treatment with collagenase is the most widely used technique for isolating stromal cells [10,12,13,18–20], this treatment varies in the literature. Trypsin or other enzymes such as hyaluronidase have been frequently but not systematically added to the collagenase, and the incubation time also varies from 4 to >24 h. Moreover, some authors have removed the cord vessels by stripping them manually before enzymatic treatment [21]. Several groups do not use enzymatic treatment [22–30], but most of them dissected the cord segment into very small pieces (1–3 mm^3), with or without discarding the cord vessels [23–30].

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In this study, we assessed the effectiveness of a simplified protocol based uniquely on the capacities of MSCs to adhere to a plastic surface without enzymatic treatment or dissection, and we compared this protocol with 2 others frequently used to isolate MSC from the WJ [10,24]. The objectives of this study were to obtain a sufficient number of MSCs from a small segment of umbilical cords and to overcome culture time, cell manipulation, and use of enzymes.

It is important to generate a clinical quantity of MSCs and retain MSC characteristics such as phenotype, clonogenic capacity, differentiation potential, and immunomodulatory function. In our study, we aimed to produce cells capable of promoting hematopoietic engraftment. Thus, the cells generated had to be able to sustain hematopoietic progenitor cell proliferation.

Materials and Methods

Samples

Human umbilical cords were collected after full-term deliveries with informed consent of the mothers. Segments from 5 to 10 cm were sectioned and conserved at room temperature into sterile phosphate-buffered saline (PBS) supplemented with penicillin/streptomycin until they were used in the laboratory (within 24 h). Ethics approval was obtained from the institutional ethics committee.

Isolation and culture of WJ MSCs

Longitudinally cut. The umbilical cord segments (5–10 cm) were sectioned longitudinally to expose the WJ. Some incisions were made on the matrix with a sterile scalpel to expose a wider area of tissue to contact with the plastic surface. The cord sections were then transferred to a 10 cm^2 Petri dish and plated for 5 days in Dulbecco's modified Eagle's medium with 1.0 g/L glucose, without L-glutamine (DMEM; Lonza) supplemented with 15% fetal bovine serum (Sigma), 2 mM L-Glutamine (Lonza), and 0.5% antibiotic–antimycotic solution (Lonza). Cultures were maintained in a humidified atmosphere with 5% CO_2 at 37°C. After 5 days, the cord segments were discarded and the medium was renewed. The cells were then expanded until they reached subconfluence (80–90%) with changing the medium every week. At subconfluence, the cells were harvested after detachment by 10 min incubation with TrypLE Select solution (Lonza), counted, and analyzed for their phenotype, clonogenic capacities, and differentiation capacities. For passages, 5×10^4 cells were replated in 75 cm^2 flask (Falcon) in the same culture conditions until subconfluence. Cells were passaged until P4. One representative WJ was passaged until P6.

We have compared this procedure with 2 others, frequently described to isolate MSCs from the WJ. Briefly, each cord segment of $\sim$15 cm ($n=7$), equally separated in 3 equal parts, was processed at the same time using the 3 methods, named "longitudinally cut," "fine pieces," and "enzymes."

Fine pieces. The cord segment, minced into very fine pieces from 1 to 2 mm^3 without removing of the vessels, was directly plated in a Petri dish.

Enzymes. The cord segment, dissected in fine pieces, was incubated overnight with a solution of collagenase D (0.2%; Roche Applied Science). The cells were then incubated for 30 min with a solution of trypsin ethylenediaminetetraacetic acid (Lonza) before washing in DMEM/15% FCS medium to stop enzyme activities. Finally, the cells were plated in a Petri dish.

After 5 days of contact with plastic surface, the cords (segment or fine pieces) were removed, the medium was replaced, and the cultures were pursued until cell subconfluence. When cells obtained with 1 of the 3 culture methods were subconfluent, the 3 were passaged. We evaluated the cumulative number of cells, their phenotype, and their clonogenic capacities.

Phenotypic analysis

Cells harvested after detachment with TrypLE Select were washed in PBS (Miltenyi Biotec) and were incubated for 20 min with the following monoclonal antibodies: CD105-FITC (Ancell corporation), CD73-PE (BD Biosciences Pharmingen), CD146-PC5 (Beckman Coulter), CD31-FITC (Miltenyi Biotec), CD34-PC5 (BD), CD45-PC7 (BD), CD144-APC (eBioscience), HLA-DR-PC5 (Beckman Coulter), CD166-PE (BD), and CD90-PE (R&D Systems Europe).

After washing with PBS, the cells were fixed with 8% formaldehyde. Data were acquired and analyzed on a MacsQuant analyzer (Miltenyi Biotec).

Multidifferentiation potential

The differentiation potential of MSCs was examined using cells harvested at P1 to P3.

Adipogenic, osteogenic, and chondrogenic differentiations were performed according to the manufacturer's instructions in adapted media (NH media, Miltenyi Biotec).

For the osteogenic differentiation, the cells were plated in DMEM in a 24-well plate at a density of 2,000 cells/well. After 24–48 h, the osteogenic medium (NH OsteoDiff Medium; Miltenyi Biotec) was added to the adherent cells. Every week, cells were fed with complete replacement of the medium. At days 7, 14, and 21, the calcium mineralization was assessed by coloration with Alizarin Red (Sigma), as described by Meloan et al. [31] with slight modifications. Cells were washed in PBS and fixed in 70% ethanol at room temperature for 5 min followed by several washes in H_2O. Cells were stained in 40 mM Alizarin Red (Sigma) pH 4.2 for 15 min at room temperature, rinsed in H_2O, and then air-dried. Red staining was examined by light microscopy. The Ca^{++} accumulation was also measured (quantitative determination). To evaluate calcium deposition, the matrix was demineralized by addition of 500 µL of 0.6 NHCl and overnight incubation at 37°C. Solutions were then collected and centrifuged at 2,000×*g* for 5 min. Calcium concentration in the supernatant was determined by colorimetry (QuantiChrom Calcium Assays Kit; BioAssay Systems) as described by the manufacturer. Briefly, 5 µL samples were combined with 200 µL calcium reagent and incubated for 5 min at room temperature. The absorbance was measured immediately after incubation at 610 nm using a plate reader (Organon Teknika Cappel Products).

For the adipogenic differentiation, 4,000 cells/well were plated in a 24-well plate in NH AdipoDiff Medium (Miltenyi Biotec). After 7, 14, and 21 days, cells were colored using Oil

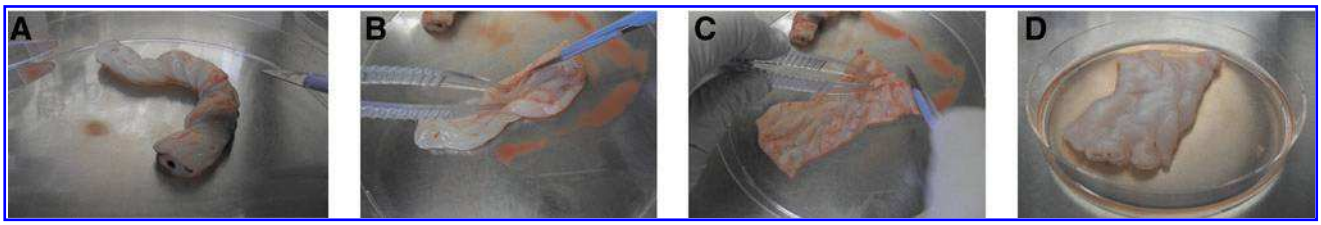

FIG. 1. The umbilical cord **(A)**, cut longitudinally **(B)**, was incised with a scalpel **(C)**. The WJ was plated and cultured in contact with plastic for 5 days of culture **(D)**. WJ, Wharton's jelly.

Red O. Briefly, cells were washed with PBS and fixed with 8% formaldehyde before staining with Oil Red O solution (Sigma).

To induce chondrogenesis, cells were transferred in the bottom of 15 mL conical tubes and differentiated into chondrocytes in pellet culture (250,000 cells/pellet) in 1 mL specific chondrocyte induction medium (NH ChondroDiff Medium; Miltenyi Biotec). Tubes were incubated for 21 days at 37°C in a 5% CO_2 incubator, and the medium was replaced every week. The pellets were then fixed with 8% formaldehyde, embedded in paraffin blocks, and cut into 4-μm-thick sections. Microtome samples were stained with Alcian Blue using standard methods.

Detection of fibroblast colony forming units (CFU-Fs)

To obtain the number of mesenchymal progenitors, CFU-Fs were evaluated at passage 1 and after each passage. Briefly, $1–5\times10^4$ cells, recovered after trypLE select detachment, were plated in a 10 cm^2 Petri dish in the MSC culture medium at 37°C in 5% CO_2 and 100% humidity. After 10 days, the medium was discarded and the adherent cells were stained with May-Grunwald Giemsa following manufacturer's instructions. Briefly, cells were covered for 5 min with May-Grunwald solution. After washing, cells were covered with Giemsa's solution for 20 min. Plates were rinsed and colonies of >50 cells were scored using an inverted microscope.

Bl-CFCs

Bl-CFCs represent a specific subpopulation of relatively primitive hematopoietic progenitors characterized by colony formation only in close contact with a preformed stromal layer. These progenitors were shown to self-renew and to give rise to multipotential and lineage-committed colony-forming progenitor cells [32,33]. This culture system allows the evaluation of the capacity of a stromal layer to sustain the survival and proliferation of early hematopoietic progenitor cells.

Briefly, 5×10^3 MSCs, obtained at P2 or P3, were plated in DMEM/fetal bovine serum medium in 4-well plates until confluence. A total of $1–5\times10^4$ CD34+ cells purified from umbilical cord blood using MidiMACS separation (Miltenyi Biotec) were added on the feeder-layer, and 1 mL semisolid culture medium without cytokine (Stemcell Technologies, Grenoble, France) was added onto the cells. Cocultures were kept at 37°C for 5–10 days. Refringent colonies of >20 cells, closely attached to the feeder layer, were counted using an inverted microscope.

Immunomodulation

The immunosuppressive effects of WJ MSCs collected at P2 were evaluated on activated T cells. Briefly, peripheral blood CD3 T lymphocytes purified by positive selection using the MACS system (Miltenyi Biotec) were stained with carboxyfluorescein diacetate succinimidyl ester and activated using PHA-IL-2 cocktail. After a 5-day incubation with different MSC concentrations, the lymphocyte proliferation was assessed by flow cytometry.

Data analysis

A total of 50 WJ samples were analyzed. Data are presented as mean ± SEM. For the direct comparison between the 3 procedures, the Student's paired *t*-test was used. All tests were 2-sided. An effect was considered to be significant at $P < 0.05$. All analyses were performed with Microsoft Excel software.

Results

Isolation of MSCs from the WJ

Contrary to the majority of groups working on WJ MSCs, we chose to process the WJ based essentially on the capacity of MSCs to adhere to a plastic surface, without enzymatic treatment, stripping of the cord vessels or dissection. We sectioned the umbilical cord segments (5–10 cm) ($n = 50$)

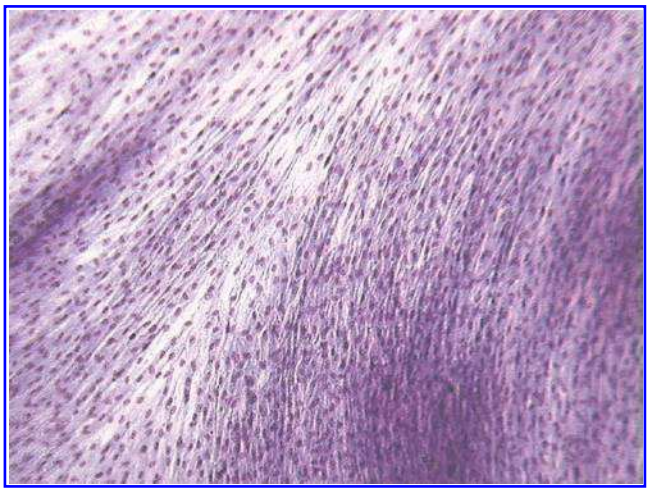

FIG. 2. Morphology of WJ MSCs. Representative sample of the typical fibroblastic-like morphology observed after the primoculture. The cells were stained using May-Grunwald-Giemsa solution. Magnification,×50. MSC, mesenchymal stromal cell.

longitudinally to expose the WJ and we made some incisions on the matrix with a sterile scalpel to expose a wider area of tissue to contact with the plastic surface. We plated then the cord sections in 10 cm^2 Petri dishes in the MSC culture medium. After 5 days of contact with plastic, the segment of umbilical cord was removed, and the medium was replaced. At this time, we observed some colonies of cells with fibroblastic morphology. The culture was pursued until cells reached subconfluence. The cells were then expanded by successive passages. This method for cell isolation is easy, fast, and reliable with low contamination risk (Fig. 1).

Characteristics of WJ cells at passage 1

After 20 ± 1 days (range, 9–35 days), a mean $\pm$ SEM of $2.72 \pm 0.33 \times 10^6$ cells (range, $0.36–10.4 \times 10^6$, $n = 50$) were recovered in a 57 cm^2 Petri dish. As shown in Fig. 2, the cells had the typical morphology of fibroblastic cells. The MSC yield reach 100%, meaning that every processed cord segment contained MSCs.

For 14 of the 50 WJ samples analyzed (<30%), the characteristic mesenchymal cell population coexisted with endothelial-like cells. Islets of rounded cells were clearly detected by microscopy analysis (Fig. 3). These islets were progressively reduced when the fibroblastic cells reached confluence. In these samples, the phenotypic analysis of cells after the primoculture revealed the presence of CD31+ cells lacking CD45, CD73, and CD90 antigen expression. At the second passage (P2), this endothelial cell population completely disappeared.

We failed to observe any correlation between the percentage of CD31+ cells at P1 and the number of cells expanded at P1 and P2 (data not shown), demonstrating that the presence of endothelial-like cells after the primoculture does not influence the proliferative capacities of MSCs.

Cell proliferative potential

An important issue of interest in the study of MSCs is the availability of the tissue and the efficacy of isolation and culture methods to obtain a sufficient number of MSCs for clinical application. Therefore, our next objective was to evaluate the efficiency of the MSC isolation protocol from the WJ and to determine the number of available cells at P3 and their functional capacities.

At P1, after 20 ± 1 days, we obtained $2.72 \pm 0.3 \times 10^6$ cells. At P2, we obtained $146 \pm 31 \times 10^6$ cells after 32 ± 1 days; at P3, the cumulative number of cells reached $7.7 \pm 2.5 \times 10^9$ cells in 45 ± 3 days (Fig. 4). The population doublings of the WJ cells, observed at P2 and P3, were, respectively, 5.21 ± 0.14 and 10.61 ± 0.36. In our experience, using BM cells under the same culture conditions, we have observed a population doubling of 1.85 ± 0.54 and 3.51 ± 0.96, at the second and the third passage, respectively.

Clonogenic capacities of cells

The clonogenic capacities of the cells were determined after each passage by evaluating the number of CFU-Fs in each cell population ($n = 50$). The mean number of CFU-Fs in the cell suspension collected at P1 was 30 ± 9 for 5,000 cells. At P2, this number was 31 ± 6 and after P3 and P4, it was 26 ± 8 and 28 ± 2, respectively. These results demonstrate the conservation of the clonogenic capacities of the WJ MSCs along the 3 first passages (Fig. 4).

To meet the criteria of the ISCT [34], we characterized the cells for different markers and differentiated the cells into osteoblasts, adipocytes, and chondroblasts to confirm their multipotentiality.

Cell phenotype

Cell expression of mesenchymal, endothelial, and hematopoietic surface markers is presented in Table 1.

At P1, <30% of the 50 WJ samples contained a cell population that expressed the CD31 antigen. Approximately 20% of these CD31+ cells coexpressed the CD144, CD146, and CD34 antigens, phenotype corresponding to endothelial progenitors. These CD31+ cells all disappeared after the second passage.

Flow cytometry analysis showed that at P2, cells were positive for the MSC markers CD105, CD73, CD90, CD166, and CD146 and negative for the hematopoietic CD45, CD34, HLA-DR, and endothelial markers CD31 and CD144 (Fig. 5).

Cell differentiation potential

Osteogenic differentiation was detected by the matrix calcification shown by Alizarin red staining. Five out 20 samples formed a mineralized matrix after 7 days of induc-

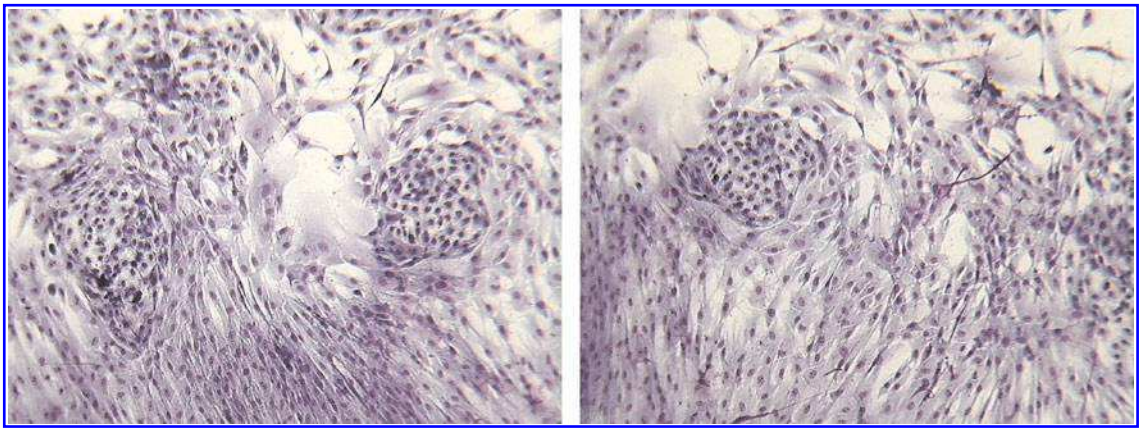

FIG. 3. Representative samples of islets of endothelial-like cells observed in the confluent MSC layer after the primoculture (magnification,×50). The cells were stained using May-Grunwald-Giemsa coloration. Color images available online at www.liebertonline.com/scd.

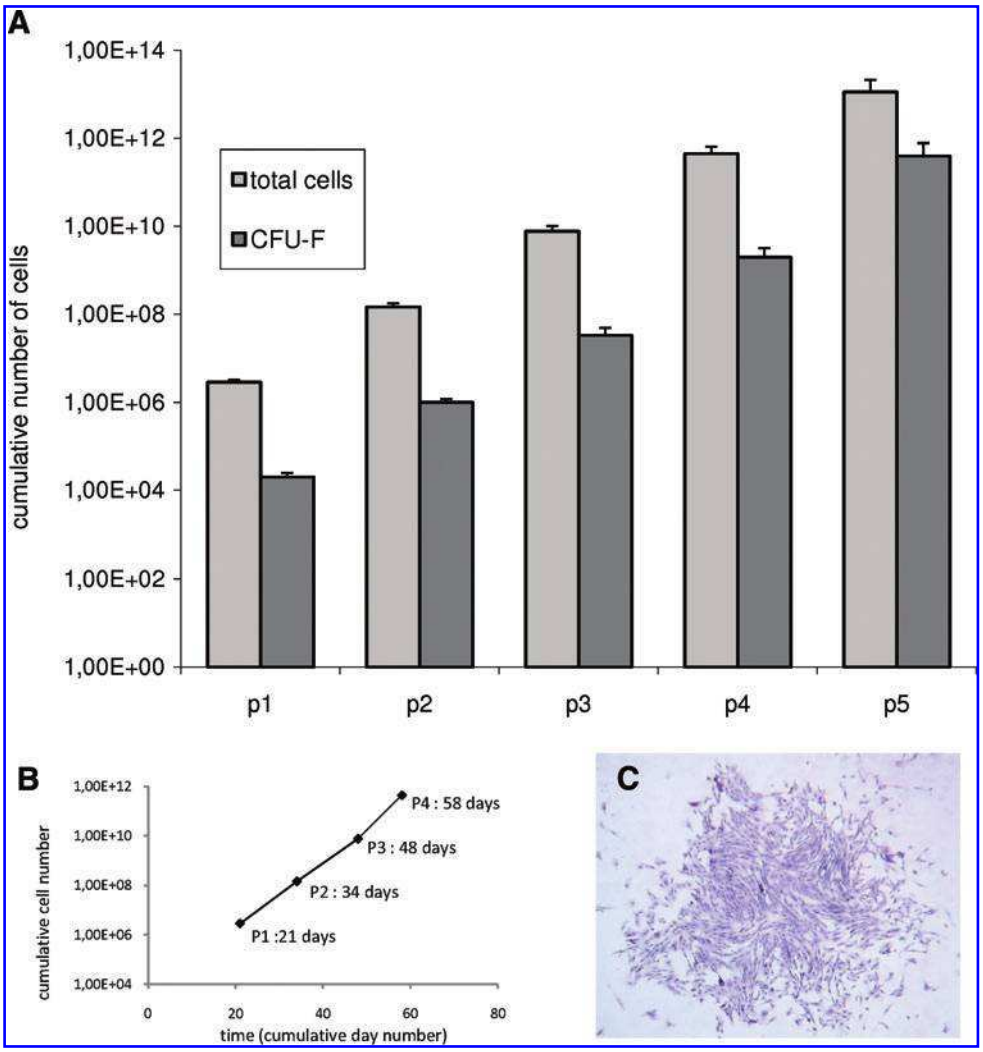

FIG. 4. **(A)** Proliferative potential of WJ cells at P1 and after passages 2–5 (■). Cumulative CFU-F numbers at P1 to P5 (■). The results are expressed as mean ± SEM of the calculated cumulative cell number ($n = 43$). **(B)** The line diagram represents the cumulative cell number versus the time. **(C)** Representative sample of 1 colony of CFU-Fs observed from MSCs obtained at P2. Color images available online at www.liebertonline.com/scd.

tion (Fig. 6A). Nine and 6 of the remaining samples formed a mineralized matrix after 14- and 21-day induction, respectively (Fig. 6B). The colorimetric dosage of Ca^{++} concentration confirmed these results (Fig. 6C).

After induction, adipogenic differentiation of WJ MSCs was observable between 7 and 14 days ($n = 20$). The cells contained a high number of very fine small lipid vacuoles stained using Oil Red O solution. This aspect was maintained after 28 days of induction (Fig. 6D, E).

To study chondrogenic differentiation, we used the pellet culture system. After 21 days of induction, pellets of 1–2 mm diameters were observed. The presence of glycosaminoglycans was shown by alcian blue staining of histological sections (Fig. 6F).

Table 1. Cell Expression of Mesenchymal, Endothelial, and Hematopoietic Surface Antigens

		CD45	*HLA-DR*	*CD34*	*CD31*	*CD105*	*CD73*	*CD146*	*CD166*	*CD90*
A ($n = 14$)	P1	2.6 ± 0.7	2.7 ± 0.7	13.8 ± 5.5	42.2 ± 7.6	69.3 ± 7.5	75 ± 7.7	76.5 ± 6.7	65.7 ± 9.0	44.6 ± 9.5
	P2	1.1 ± 0.4	2.1 ± 0.4	0.3 ± 0.1	2.4 ± 0.9	83.6 ± 9.4	84.9 ± 9.5	80.9 ± 9.8	77.1 ± 9.9	85.9 ± 9.8
B ($n = 36$)	P1	3.6 ± 1.2	1.9 ± 0.3	2.0 ± 0.5	3.6 ± 1.2	75.7 ± 3.0	84.4 ± 2.6	68.5 ± 4.0	68.5 ± 5.5	85.6 ± 3.3
	P2	0.2 ± 0.0	2.1 ± 0.3	1.4 ± 0.3	1.7 ± 0.4	86.8 ± 3.2	92.3 ± 2.1	82.2 ± 3.7	79.4 ± 5.7	86.2 ± 4.1
A+B ($n = 50$)	P3	1.86 ± 1.2	3.0 ± 0.8	1.3 ± 0.3	1.3 ± 0.2	81 ± 2.9	90.7 ± 2	78 ± 4	80.8 ± 3	86 ± 3.6
	P4	2.3 ± 10.2	1.5 ± 0.5	0.8 ± 0.3	2.9 ± 1.2	83 ± 6.6	95.3 ± 1.3	76.7 ± 6.8	84.4 ± 6.2	80.2 ± 9.2

Percentages of positive cells at passages 1–4. Results are expressed as mean ± SEM. A represents samples with endothelial-like cells at P1; B represents samples with <10% of endothelial like cells at P1. For the percentages of positive cells after P3 and P4, the 2 series of samples are pooled because the percentages are similar.

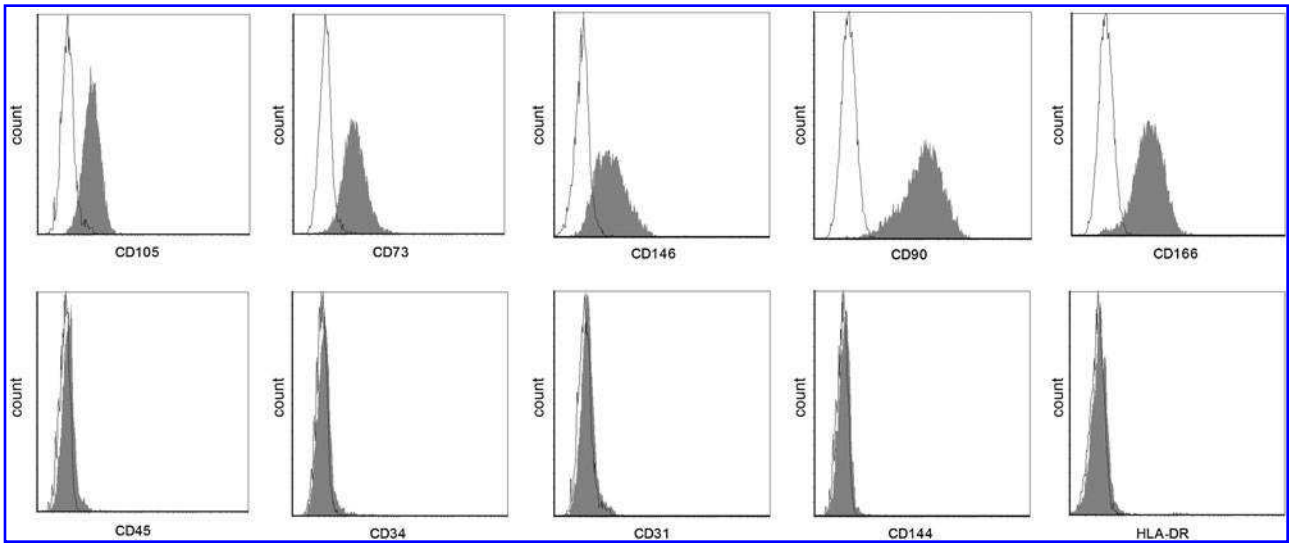

FIG. 5. Flow cytometric analysis of surface marker expression on WJ MSCs. The data shown are the representative cell phenotype analyzed at P2.

Immunomodulation

To determine whether WJ MSCs affect the proliferation of T cells in vitro, we evaluated the percentage of mitogen-induced proliferating T cells by carboxyfluorescein diacetate succinimidyl ester labeling. When PHA-activated $CD3^+$ cells were incubated on MSCs, T cell proliferation decreased significantly. This effect was dependent on the number of MSCs in the coculture ($n = 12$) (Fig. 7).

When added to a mixed lymphocyte reaction (MLR), the WJ MSCs actively suppressed the allogeneic proliferation of responder lymphocytes, also in a ratio-dependent manner (data not shown).

BI-CFC assay

As a final step, we evaluated the capacities of confluent layers of WJ MSCs to sustain the proliferation of blast-

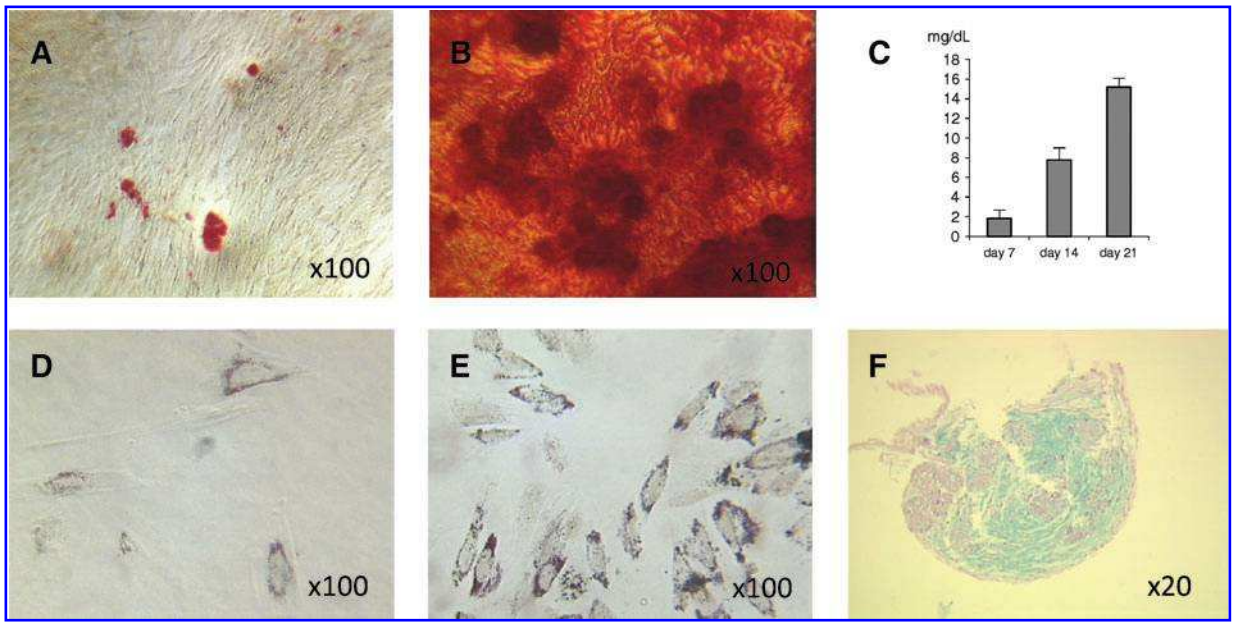

FIG. 6. **(A, B)** Representative sample of the differentiation into osteoblasts after 7 days of induction **(A)** and after 21 days of induction **(B)**. The calcium mineralization was assessed by Alizarin Red staining. **(C)** The histogram represents the calcium concentration in the supernatant determined by colorimetry. Results are the mean of 5 experiments. **(D, E)** Representative sample of the differentiation into adipocytes after 7 days of induction **(D)** and after 28 days of induction **(E)**. Cells were colored using Oil Red O. **(F)** Representative sample of the differentiation into chondrocytes after 21 days of induction. Microtome sample was stained with Alcian Blue.

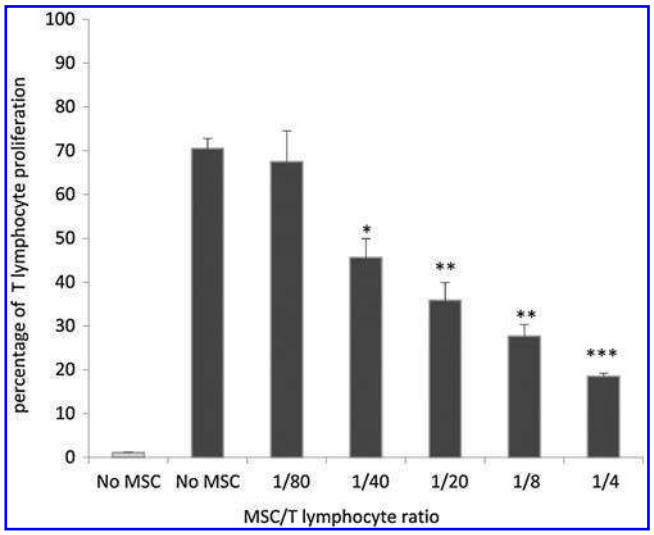

FIG. 7. Decrease in T lymphocyte cell proliferation when PHA-activated CD3+ cells were incubated on increased ratio of MSCs. The number of mitogen-induced proliferating T cells was determined by carboxyfluorescein diacetate succinimidyl ester labeling. Results are expressed as mean percentage ± SEM of proliferative T cells ($n = 12$) in (■) stimulated cells and (■) unstimulated cells. The positive control is represented by the proliferation percentage of stimulated T lymphocytes and the negative control by the proliferation of unstimulated T lymphocytes, both in absence of MSCs. The proliferation of unstimulated T cells was not modulated by the presence of MSCs (data not shown). Significant difference between the positive control and the cells with increased ratio of MSCs was calculated using the Student's paired *t*-test: $*P < 0.02$, $**P < 0.002$, $***P < 0.0001$.

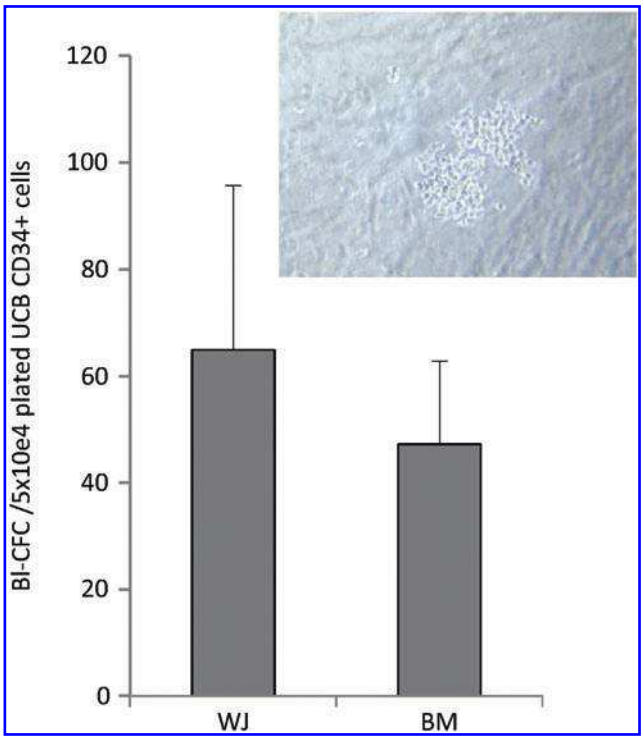

FIG. 8. Number of Bl-CFC per 5×10^4 cord blood $CD34^+$ on WJ and bone marrow confluent stromal layer. Results are expressed as mean ± SEM ($n = 9$). (*Inset*) Representative sample of Bl-CFC colonies observed after 5 days of incubation of UCB $CD34^+$ cells on a confluent stromal layer of WJ cells. Magnification, ×100. Color images available online at www.liebertonline.com/scd.

colony-forming cells issued from UCB CD34+ cells ($n = 9$) and we compared them with those of BM MSCs. CD34+ cells were plated either on confluent WJ feeder layers or on confluent BM feeder layers in the semisolid culture medium for 5 days. After this time period, colonies of refringent cells strongly attached to the MSCs were scored (Fig. 8). A mean of 64 ± 30 Bl-CFCs per 5×10^4 CD34+ cells was observed in the WJ coculture and 47 ± 15 in the BM coculture, demonstrating that the feeder layer constituted by WJ MSCs could sustain the attachment and the proliferation of hematopoietic progenitor cells without the addition of exogenous cytokines.

Direct comparison of the 3 procedures to isolate MSCs from the WJ

Seven cord segments of ~15 cm, equally separated in 3 equal parts, were processed in the same time with the 3 methods, named "longitudinally cut," "fine pieces," and "enzymes." The first one is described before. For the second, the cord segment, minced into very fine pieces from 1 to 2 mm^3 without removing of the vessels, was directly plated in Petri dish. For the third, the cord segment, dissected in fine pieces, was treated with collagenase and trypsin before plating. After 5 days of contact with plastic surface, the cords (segment or fine pieces) were removed, the medium was replaced, and the cultures were pursued until cell subconfluence. When cells processed with 1 of the 3 culture methods were subconfluent, the 3 were passaged. We evaluated the cumulative number of cells, their phenotype, and their clonogenic capacities, as well as the duration of the cell process.

When we compared the duration of the laboratory procedures for cell isolation before plating, it appears clearly that the procedure named "longitudinally cut" was faster (a mean of 2 min 34 s) than the 2 others (respectively, fine pieces and enzymes take a mean of 8 min 58 s and 20 h 56 min 50 s). Although at P1 the cell number is significantly lower using "enzymes" procedure, at P2 and P3 the cumulative cell numbers as well as their clonogenic capacities are not significantly different using the 3 methods (Fig. 9). In the same way, the phenotype of the cells and their induction capacities are comparable using the 3 methods (data not shown).

Discussion

Cord blood has been used since 1988 as a transplantable source of hematopoietic stem cells [35]. Unfortunately, despite many advantages (availability, lower histocompatibility requirement, etc.), the amount of cells collected in a cord blood sample is limited, leading to delayed engraftment, and, in most cases, is sufficient only for child recipients [2]. This limitation may be overcome through the co-infusion of cord blood samples with MSCs, known to support hematopoiesis. Indeed, previous studies have shown that the cotransplantation

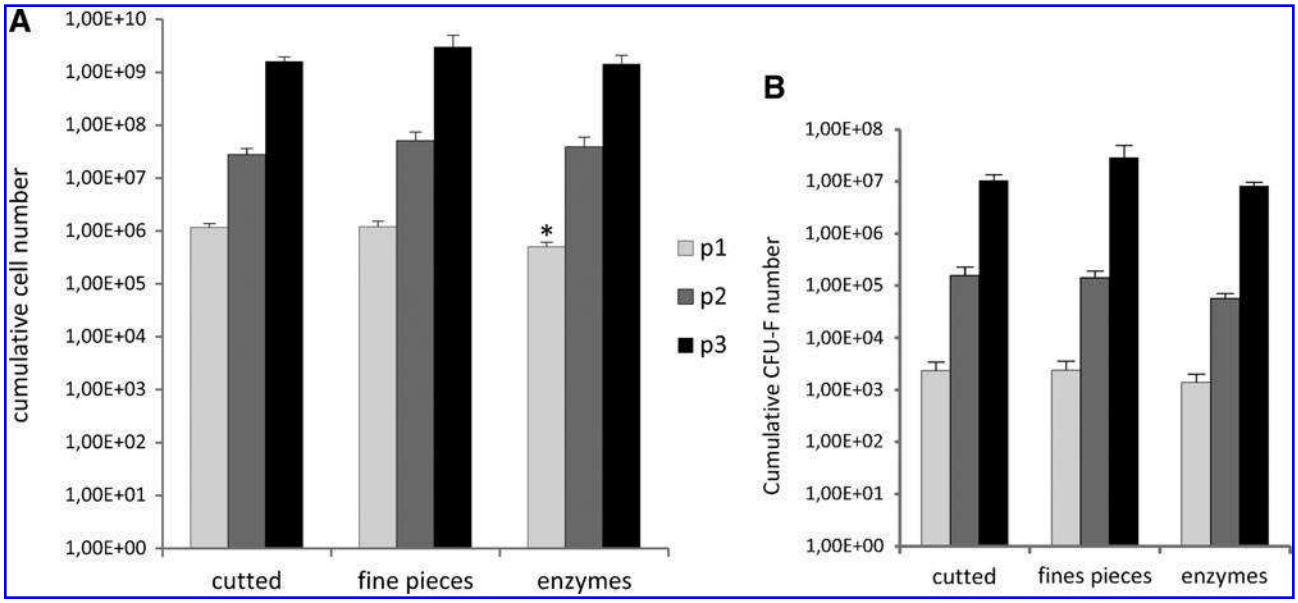

FIG. 9. Comparison between the cell proliferation **(A)** and the CFU-F number **(B)** observed using 3 different procedures for MSC isolation. The first one described in this article was named "longitudinally cut," the 2 others were named "fine pieces" and "enzymes." Five cord segments were simultaneously treated using the 3 procedures. Results are expressed as mean SEM of the cumulative cell number obtained after P1, P2, and P3. The Student's paired *t*-test was used to compare the cell number of "fine piece" and "enzymes" procedures with the "longitudinally cut" procedure. $^*P < 0.03$.

of hematopoietic stem cells with MSCs could enhance hematopoietic engraftment in mice [36] and in humans [6,37–40].

Several sources of MSCs can be envisaged. Among them, BM and adipose tissue are the most currently described and used in clinical settings [41]; however, their collection procedures are relatively invasive and are usually limited to adult donors. It was previously described that extra embryonic tissues, usually discarded at birth, including umbilical cord blood [42,43] and WJ [44], may be considered as potential sources for MSCs. These tissues have several advantages, including accessibility, painless procedures to donors, possible source for autologous cell therapy, and lower risk of contamination. In cord blood, the possibility to isolate sufficient numbers of MSCs for clinical applications remains controversial. Moreover, several studies have shown that the number of MSCs in 1 cord blood sample is relatively low and that the MSCs have very low cell proliferative activity [45]. In addition, MSCs only appear to be present in a small percentage of cord blood samples [46]. On the contrary, in the WJ, several studies have demonstrated the presence of MSCs in 100% of the samples studied [19,46,47]. The WJ thus appears to be particularly attractive; however, there is no standardized system to isolate MSCs from this tissue. This lack of a standardized system encouraged us to explore a rapid, easy, and efficient isolation procedure for the isolation of MSCs from the WJ. To date, most groups have used enzymatic treatment, using several proteases such as collagenase, hyaluronidase, or trypsin associated with or without mechanical removal of the vessels [10,12,13,18,20,21]. However, it is known that the overdigestion of tissue may result in diminished cellular viability, degradation of cellular surface receptors, and altered cellular function [48]. Moreover, the use of enzymes from nonhuman species would be avoided for the preparation of cells destined for clinical use.

The possibility to obtain MSCs from the umbilical cord matrix by a nonenzymatic isolation procedure was first described by La Rocca et al. [22], followed by other groups [23–30]. Again, however, the procedure has yet to be standardized. While La Rocca et al. reduced the cord use into segments of 1–2 cm, sectioning the segments longitudinally and plating them into wells for 15 days, other groups have dissected the cord segments into very small pieces (1–3 mm^3) before plating, which could lead to a higher risk of contamination and a longer procedure in the laboratory.

Here, we describe a simplified method to that described by La Rocca et al. [22]. We used 5–10-cm segments for each cord analyzed. These segments were sectioned longitudinally and plated for 5 days on a plastic surface. The isolation method, based on the migratory and plastic adhesion capacities of MSCs, does not use enzymatic treatment or dissection of the tissue.

Using this method, we were able to successfully isolate MSCs from all human full-term umbilical cords analyzed. These findings are consistent with studies using enzymatic treatment, in which MSC yield was reported to be 100% [12,16,18], meaning that each cord processed contained MSCs.

Using our method, we observed some residual endothelial contamination at P1 in 30% of the WJ samples analyzed. This contamination disappeared after the second passage and did not influence the proliferation capacities of the MSCs or their phenotypes, suggesting that the removal of vessels and arteries at the start of the culture is not essential and may be avoided considering the high risk of contamination induced by this procedure. Further, cells expressing mesenchymal markers such as CD44, CD105, CD73, and CD90 are present in situ in both the WJ and umbilical cord vessels [25], and the isolation of MSCs from the subendothelium layer of

the umbilical cord vein is feasible [44]. Removal of the vessels could lead to the loss of these MSCs.

Consistent with previously described work using MSCs isolated by enzymatic treatment, the cells obtained using our method were able to differentiate into osteoblasts, adipocytes, and chondrocytes. However, conflicting data concerning the differentiation capacities of WJ cells in adipocytes and osteoblasts have been reported [12,49,50]; these conflicts are likely due to the heterogeneity between the MSC selection procedures. In our study, the differentiation into osteoblasts occurred after 7–14 days of induction. This relatively rapid differentiation seems discordant with several studies showing that the differentiation of WJ MSCs into osteoblasts is difficult and slow [51,52]. However, our results are consistent with others [18,53] who showed that perivascular umbilical cord cells can form bone nodules after a 5-day induction period [18].

Similar to osteoblasts, the majority of MSCs differentiated into adipocytes in 7 days. However, the differentiated cells contained very fine lipid vacuoles. This aspect of relatively immature adipocytes was maintained after 14, 21, and 28 days of induction, suggesting that the adipocytes derived from the WJ MSCs mature relatively slowly under our induction conditions. These results are consistent with previous data described on WJs [54] and on cord blood MSCs [55]. WJ MSCs seem thus more primitive cells sharing common genes with embryonic stem cells, as recently reported by Hsieh et al. [52].

In addition to their differentiation capacities, the ability of the WJ MSCs to modulate the immune response was also investigated. Our results demonstrated that the MSCs isolated using our procedure were able to inhibit the proliferation of lymphocytes T cells in a similar way to what previously shown using BM MSCs [56]. These results are consistent with what has been described using MSCs obtained by enzymatic treatment [16]. Finally, we confirmed that the WJ cells obtained using our procedure were able to support the in vitro proliferation of early hematopoietic cells in the same way that BM MSCs.

Finally, we determined whether the isolation of a sufficient number of MSCs was possible using our procedure. The estimated number of MSCs for clinical applications ranges from 1 to 2×10^6 cells/kg body weight [37]. In this study, we obtained a mean of 2.8×10^6 cells with cord segments of 5–10 cm at P1 (20 days). At P2, the number of cells reached $>1.4\times10^8$ (32 days) and $>7\times10^9$ cells at P3 (45 days). Our results demonstrated that at P2, the number of MSCs was already sufficient to transplant into an adult. These results are in the same range as those observed using enzymatic treatment, even if these last ones were very heterogeneous following the authors. Using enzymatic treatment on 1 mm^3 fragments, Lu et al. obtained a mean number of 2.6×10^7 cells with a 30 cm segment of umbilical cord after the primary culture [19]. Nekanti et al., using enzymatic treatment and removal of the vessels, obtained 5–6×10^4 cells per cord after the primary culture [21]. Finally, using enzymatic treatment and the removal of vessels, Karahuseyinoglu et al. obtained a total of 3.6×10^6 viable cells per 15 cm of umbilical cord after the primary culture. In this study, the total cell number reached 11.5×10^8 in 7 months [12]. Surprisingly, while the number of cells after the primary culture was comparable to those observed in our study, the time to obtain $>10^8$ cells was much longer, possibly due either to the enzymatic treatment or to different culture conditions.

To validate our method, we have compared it directly with 2 others frequently used to isolate the WJ MSCs [10,24]. Our method takes significantly less time to process the cord and the simplification of the manipulations leads to a decrease of contamination risks. Moreover, we do not show any significant difference between the 3 methods in terms of cell proliferation and clonogenic capacities of cells after P1.

In conclusion, we have demonstrated that it is possible to obtain a sufficient number of MSCs from 5 to 10 cm cord segments, without enzymatic treatment and tissue dissection, after only 5 days of contact with plastic and 30 days of culture. We have shown that this protocol is reproducible, is simple, and can be quickly performed. Moreover, we have shown that the obtained MSCs expressed characteristic markers and had typical functional properties of MSCs, including adhesion to plastic, differentiation capacities, and immunological properties. Most importantly, these cells were able to sustain in vitro proliferation of hematopoietic progenitor cells.

Knowing that the umbilical cord reaches an average length of 50–60 cm at the birth, $\sim7\times10^8$ to 14×10^8 cells could be obtained from 1 umbilical cord after 1 month of culture. Regarding the relatively high proliferation capacities of the WJ MSCs, the WJ could be envisaged not only to sustain hematopoiesis during cord blood transplant but also to be a valuable source of MSCs in the field of regenerative medicine.

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Author Disclosure Statement

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References

1. Gluckman E. (2009). History of cord blood transplantation. Bone Marrow Transplant 44:621–626.
2. Wagner JE and E Gluckman. (2010). Umbilical cord blood transplantation: the first 20 years. Semin Hematol 47:3–12.
3. Noort WA, AB Kruisselbrink, PS In't Anker, M Kruger, RL van Bezooijen, RA de Paus, MH Heemskerk, CW Löwik, JH Falkenburg, R Willemze and WE Fibbe. (2002). Mesenchymal stem cells promote engraftment of human umbilical cord blood-derived CD34(+)cells in NOD/SCID mice. Exp Hematol. 30:870–878.
4. Angelopoulou M, E Novelli, JE Grove, HM Rinder, C Civin, L Cheng and DS Krause. (2003). Cotransplantation of human mesenchymal stem cells enhances human myelopoiesis and megakaryocytopoiesis in NOD/SCID mice. Exp Hematol 31:413–420.
5. Bakhshi T, RC Zabriskie, S Bodie, S Kidd, S Ramin, LA Paganessi, SA Gregory, HC Fung and KW Christopherson. (2008). Mesenchymal stem cells from the Wharton's jelly of umbilical cord segments provide stromal support for the

maintenance of cord blood hematopoietic stem cells during long-term *ex vivo* culture. Transfusion 48:2638–2644.
6. Gonzalo-Daganzo R, C Regidor, Martín-Donaire T, MA Rico, G Bautista, I Krsnik, R Forés, E Ojeda, I Sanjuán, JA García-Marco, B Navarro, S Gil, R Sánchez, N Panadero, Y Gutiérrez, M García-Berciano, N Pérez, I Millán, R Cabrera and MN Fernández. (2009). Results of a pilot study on the use of third-party donor mesenchymal stromal cells in cord blood transplantation in adults. Cytotherapy 11:278–288.
7. Sensebé L and P Bourin. (2009). Mesenchymal stem cells for therapeutic purposes. Transplantation. 87:S49–S53.
8. Bajada S, I Mazakova, JB Richardson and N Ashammakhi. (2008). Updates on stem cells and their applications in regenerative medicine. J Tissue Eng Regen Med 2:169–183.
9. McElreavey KD, AI Irvine, KT Ennis and WH McLean. (1991). Isolation, culture and characterisation of fibroblast-like cells derived from the Wharton's jelly portion of human umbilical cord. Biochem Soc Trans 19:29S.
10. Wang HS, SC Hung, ST Peng, CC Huang, HM Wei, YJ Guo, YS Fu, MC Lai and CC Chen. (2004). Mesenchymal stem cells in the Wharton's jelly of the human umbilical cord. Stem Cells 22:1330–1337.
11. Can A and S Karahuseyinoglu. (2007). Concise review: human umbilical cord stroma with regard to the source of fetus-derived stem cells. Stem Cells 25:2886–2895.
12. Karahuseyinoglu S, O Cinar, E Kilic, F Kara, GG Akay, DO Demiralp, A Tukun, D Uckan and A Can. (2007). Biology of stem cells in human umbilical cord stroma: *in situ* and *in vitro* surveys. Stem Cells 25:319–331.
13. Pereira WC, I Khushnooma, M Madkaikar and K Ghosh. (2008). Reproducible methodology for the isolation of mesenchymal stem cells from human umbilical cord and its potential for cardiomyocyte generation. J Tissue Eng Regen Med 2:394–399.
14. Girdlestone J, VA Limbani, AJ Cutler and CV Navarrete. (2009). Efficient expansion of mesenchymal stromal cells from umbilical cord under low serum conditions. Cytotherapy 11:738–748.
15. Hou T, J Xu, X Wu, Z Xie, F Luo, Z Zhang and L Zeng. (2009). Umbilical cord Wharton's Jelly: a new potential cell source of mesenchymal stromal cells for bone tissue engineering. Tissue Eng Part A 15:2325–2334.
16. Weiss ML, C Anderson, S Medicetty, KB Seshareddy, RJ Weiss, I Vander Werff, D Troyer and KR McIntosh. (2008). Immune properties of human umbilical cord Wharton's jelly-derived cells. Stem Cells 26:2865–2874.
17. Troyer DL and ML Weiss. (2008). Wharton's jelly-derived cells are a primitive stromal cell population. Stem Cells 26:591–599.
18. Sarugaser R, D Lickorish, D Baksh, MM Hosseini and JE Davies. (2005). Human umbilical cord perivascular (HUCPV) cells: a source of mesenchymal progenitors. Stem Cells 23:220–229.
19. Lu LL, YJ Liu, SG Yang, QJ Zhao, X Wang, W Gong, ZB Han, ZS Xu, YX Lu, D Liu, ZZ Chen and ZC Han. (2006). Isolation and characterization of human umbilical cord mesenchymal stem cells with hematopoiesis-supportive function and other potentials. Haematologica 91:1017–1026.
20. Kadam SS, S Tiwari and RR Bhonde. (2009). Simultaneous isolation of vascular endothelial cells and mesenchymal stem cells from the human umbilical cord. In Vitro Cell Dev Biol Anim 45:23–27.
21. Nekanti U, VB Rao, AG Bahirvani, M Jan, S Totey and M Ta. (2010). Long-term expansion and pluripotent marker array analysis of Wharton's jelly-derived mesenchymal stem cells. Stem Cells Dev.19:117–30.
22. La Rocca G, R Anzalone, S Corrao, F Magno, T Loria, Lo Iacono M, Di Stefano A, P Giannuzzi, L Marasà, F Cappello, G Zummo and F Farina. (2009). Isolation and characterization of Oct-4+/HLA-G+ mesenchymal stem cells from human umbilical cord matrix: differentiation potential and detection of new markers. Histochem Cell Biol 131:267–282.
23. Wu LF, NN Wang, YS Liu and X Wei. (2009). Differentiation of Wharton's jelly primitive stromal cells into insulin-producing cells in comparison with bone marrow mesenchymal stem cells. Tissue Eng Part A 15:2865–2873.
24. Petsa A, S Gargani, A Felesakis, N Grigoriadis and I Grigoriadis. (2009). Effectiveness of protocol for the isolation of Wharton's Jelly stem cells in large-scale applications. In Vitro Cell Dev Biol Anim 45:573–576.
25. Ishige I, Nagamura-Inoue T, MJ Honda, R Harnprasopwat, M Kido, M Sugimoto, H Nakauchi and A Tojo. (2009). Comparison of mesenchymal stem cells derived from arterial,venous, and Wharton's jelly explants of human umbilical cord. Int J Hematol 90:261–269.
26. Mitchell KE, ML Weiss, BM Mitchell, P Martin, D Davis, L Morales, B Helwig, M Beerenstrauch, K Abou-Easa, T Hildreth, D Troyer and S Medicetty. (2003). Matrix cells from Wharton's jelly form neurons and glia. Stem Cells 21:50–60.
27. Penolazzi L, R Vecchiatini, S Bignardi, E Lambertini, E Torreggiani, A Canella, T Franceschetti, G Calura, F Vesce and R Piva. (2009). Influence of obstetric factors on osteogenic potential of umbilical cord-derived mesenchymal stem cells. Reprod Biol Endocrinol 7:106–112.
28. Moretti P, T Hatlapatka, D Marten, A Lavrentieva, I Majore, R Hass and C Kasper. (2009). Mesenchymal stromal cells derived from human umbilical cord tissues: primitive cells with potential for clinical and tissue engineering applications. Adv Biochem Eng Biotechnol 123:29–54.
29. Magin AS, NR Körfer, H Partenheimer, C Lange, A Zander and T Noll. (2009). Primary cells as feeder cells for coculture expansion of human hematopoietic stem cells from umbilical cord blood—a comparative study. Stem Cells Dev 18:173–186.
30. Valencic E, E Piscianz, M Andolina, A Ventura and A Tommasini. (2010). The immunosuppressive effect of Wharton's jelly stromal cells depends on the timing of their licensing and on lymphocyte activation. Cytotherapy 12:154–160.
31. Meloan SN, H Puchtler and LS Valentine. (1972). Alkaline and acid alizarin red S stains for alkali-soluble and alkali-insoluble calcium deposits. Arch Pathol 93:190–197.
32. Metcalf D. (1998). Pre-progenitor cells: a proposed new category of hematopoietic precursor cells. Leukemia 12:1–3.
33. Gordon MY, CR Dowding, GP Riley and MF Greaves. (1987). Characterisation of stroma-dependent blast colony-forming cells in human marrow. J Cell Physiol 130:150–156.
34. Dominici M, K Le Blanc, I Mueller, I Slaper-Cortenbach, F Marini, D Krause, R Deans, A Keating, DJ Prockop and E Horwitz. (2006). Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. Cytotherapy 8:315–317.
35. Gluckman E, HA Broxmeyer, AD Auerbach, HS Friedman, GW Douglas, A Devergie, H Esperou, D Thierry, G Socie and P Lehn. (1989). Hematopoietic reconstitution in a patient with Fanconi's anemia by means of umbilical-cord blood from an HLA-identical sibling. N Engl J Med 321:1174–1178.

36. Friedman R, M Betancur, L Boissel, H Tuncer, C Cetrulo and H Klingemann. (2007). Umbilical cord mesenchymal stem cells: adjuvants for human cell transplantation. Biol Blood Marrow Transplant 13:1477–1486.
37. Le Blanc K, H Samuelsson, B Gustafsson, M Remberger, B Sundberg, J Arvidson, P Ljungman, H Lönnies, S Nava and O Ringdén. (2007). Transplantation of mesenchymal stem cells to enhance engraftment of hematopoietic stem cells. Leukemia 21:1733–1738.
38. Kim DW, YJ Chung, TG Kim, YL Kim and IH Oh. (2004). Cotransplantation of third-party mesenchymal stromal cells can alleviate single-donor predominance and increase engraftment from double cord transplantation. Blood 103:1941–1948.
39. Macmillan ML, BR Blazar, TE DeFor and JE Wagner. (2009). Transplantation of *ex-vivo* culture-expanded parental haploidentical mesenchymal stem cells to promote engraftment in pediatric recipients of unrelated donor umbilical cord blood:results of a phase I-II clinical trial. Bone Marrow Transplant 43:447–454.
40. Lazarus HM, ON Koc, SM Devine, P Curtin, RT Maziarz, HK Holland, EJ Shpall, P McCarthy, K Atkinson, BW Cooper, SL Gerson, MJ Laughlin, FR Loberiza Jr., AB Moseley and A Bacigalupo. (2005). Cotransplantation of HLA-identical sibling culture-expanded mesenchymal stem cells and hematopoietic stem cells in hematologic malignancy patients. Biol Blood Marrow Transplant 11:389–398.
41. Mosna F, L Sensebé and M Krampera. (2010). Human bone-marrow and adipose tissue mesenchymal stem cells: a user's guide. Stem Cells Dev 19:1449–1470.
42. Bieback K and H Klüter. Mesenchymal stromal cells from umbilical cord blood. (2007). Curr Stem Cell Res Ther 2:310–323.
43. Harris DT. (2009). Non-haematological uses of cord blood stem cells. Br J Haematol 147:177–184.
44. Romanov YA, VA Svintsitskaya and VN Smirnov. (2003). Searching for alternative sources of postnatal human mesenchymal stem cells: candidate MSC-like cells from umbilical cord. Stem Cells 21:105–110.
45. Musina RA, ES Bekchanova, AV Belyavskii, TS Grinenko and GT Sukhikh. (2007). Umbilical cord blood mesenchymal stem cells. Bull Exp Biol Med 143:127–131.
46. Zeddou M, A Briquet, B Relic, C Josse, MG Malaise, A Gothot, C Lechanteur and Y Beguin. (2010). The umbilical cord matrix is a better source of mesenchymal stem cells (MSC) than the umbilical cord blood. Cell Biol Int 34:693–701.
47. Secco M, E Zucconi, NM Vieira, LL Fogaça, A Cerqueira, MD Carvalho, T Jazedje, OK Okamoto, AR Muotri and M Zatz. (2008). Multipotent stem cells from umbilical cord: cord is richer than blood! Stem Cells 26:146–150.
48. Hung CT and RL Mauck. (2004). Biological assays: cellular level. In: *Biomedical Technology and Devices Handbook*. Moore JE, G Zouridakis, eds. CRS Press, London, pp 15:1–15:39.
49. Shetty P, K Cooper and C Viswanathan. (2010). Comparison of proliferative and multilineage differentiation potentials of cord matrix, cord blood, and bone marrow mesenchymal stem cells. Asian J Transfus Sci 4:14–24.
50. Wang L, I Tran, K Seshareddy, ML Weiss and MS Detamore. (2009). A comparison of human bone marrow-derived mesenchymal stem cells and human umbilical cord-derived mesenchymal stromal cells for cartilage tissue engineering. Tissue Eng Part A 8:2259–2266.
51. Zhang ZY, SH Teoh, MS Chong, JT Schantz, NM Fisk, MA Choolani and J Chan. (2009). Superior osteogenic capacity for bone tissue engineering of fetal compared with perinatal and adult mesenchymal stem cells. Stem Cells 27:126–137.
52. Hsieh JY, YS Fu, SJ Chang, YH Tsuang and HW Wang. (2010). Functional module analysis reveals differential osteogenic and stemness potentials in human mesenchymal stem cells from bone marrow and Wharton's jelly of umbilical cord. Stem Cells Dev [Epub ahead of print].
53. Baksh D, R Yao and RS Tuan. (2007). Comparison of proliferative and multilineage differentiation potential of human mesenchymal stem cells derived from umbilical cord and bone marrow. Stem Cells 25:1384–1392.
54. Karahuseyinoglu S, C Kocaefe, D Balci, E Erdemli and A Can. (2008). Functional structure of adipocytes differentiated from human umbilical cord stroma-derived stem cells. Stem Cells 26:682–691.
55. Rebelatto CK, AM Aguiar, MP Moretão, AC Senegaglia, P Hansen, F Barchiki, J Oliveira, J Martins, C Kuligovski, F Mansur, A Christofis, VF Amaral, PS Brofman, S Goldenberg, LS Nakao and A Correa. (2008). Dissimilar differentiation of mesenchymal stem cells from bone marrow, umbilical cord blood, and adipose tissue. Exp Biol Med 233:901–913.
56. Najar M, G Raicevic, HI Boufker, HF Kazan, C De Bruyn, N Meuleman, D Bron, M Toungouz and L Lagneaux. (2010). Mesenchymal stromal cells use PGE2 to modulate activation and proliferation of lymphocyte subsets: combined comparison of adipose tissue, Wharton's jelly and bone marrow sources. Cellular Immunology 264:171–179.

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