Optimizing isolation culture and freezing methods to preserve Wharton's jelly's mesenchymal stem cell (MSC) properties: an MSC banking protocol validation for the Hellenic Cord Blood Bank

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BACKGROUND: Mesenchymal stem or stromal cells (MSCs) are a heterogeneous population that can be isolated from many tissues including umbilical cord Wharton's jelly (UC-WJ). Although initially limited in studies such as a hematopoietic stem cell transplantation adjuvant, an increasing number of clinical trials consider MSCs as a potential anti-inflammatory or a regenerative medicine agent. It has been proposed that creating a repository of MSCs would increase their availability for clinical applications. The aim of this study was to assess the optimal isolation and cryopreservation procedures to facilitate WJ MSC banking.

STUDY DESIGN AND METHODS: Cells were isolated from UC-WJ using enzymatic digestion or plastic adhesion methods. Their isolation efficacy, growth kinetics, immunophenotype, and differentiation potential were studied, as well as the effects of freezing. Flow cytometry for common MSC markers was performed on all cases and differentiation was shown with histocytochemical staining. Finally, the isolation efficacy on cryopreserved WJ tissue fragments was tested.

RESULTS: MSC isolation was successful using both isolation methods on fresh UC-WJ tissue. However, UC-WJ MSC isolation from frozen tissue fragments was impossible. Flow cytometry analysis revealed that only MSC markers were expressed on the surface of the isolated cells while differentiation assays showed that they were capable of trilinear differentiation. All the above characteristics were also preserved in isolated UC-WJ MSCs over the cryopreservation study period.

CONCLUSION: These data showed that viable MSCs can only be isolated from fresh UC-WJ tissue, setting the foundation for clinical-grade banking.

Mesenchymal stem or stromal cells (MSCs) have been initially known for forming the hematopoietic supportive stroma of the bone marrow.[1,2] MSCs can also be isolated from different sources such as adipose tissue,[3] amniotic fluid,[4,5] umbilical cord (UC) blood,[6-8] the umbilical vein,[9] and UC's Wharton's jelly (WJ).[10,11] All these alternative sources for MSCs have been discovered in the past 20 years, but scientific knowledge is developing significantly in this rapidly evolving field. Even though many of the original studies denoted that the MSCs obtained from different tissues have the same characteristics as their marrow-isolated counterparts,[3,4,12] more recent ones indicate that there might be differences on the proliferative capacity[13] or even the therapeutic potential of the cells,[13,14] depending on their origin.

ABBREVIATIONS: CDT(s) = cell doubling time(s); FP-I (-II) = frozen Primoculture I (II) cells; HSC(s) = hematopoietic stem cell(s); MSC(s) = mesenchymal stem or stromal cell(s); P-I (-II) = Primoculture I (II); UC-WJ = umbilical cord Wharton's jelly.

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In general, MSCs have the capacity to differentiate in vitro and in vivo into several mesenchymal tissues, such as bone, cartilage, and fat, and can migrate and engraft into injured tissues.[15,16] These characteristics make MSCs an interesting regenerative medicine tool. Recent advances involve their use in tissue regeneration[17-21] and wound healing[22,23] either through a trophic effect[24,25] exerted by the MSCs in site of injury or through tissue engineering applications.[26-28] Moreover, their immunomodulatory properties have placed them into focus for the treatment of immune-related conditions such as multiple sclerosis,[29] Type 1 diabetes mellitus,[30] and Crohn's disease.[31] Most importantly, since the first case report in 2004 describing durable remission of steroid-resistant acute graft-versus-host disease (GVHD)[32] after MSCs infusion, multiple Phase II clinical trials[33-36] have provided a strong rationale for what now tends to become a routine medical practice in many European jurisdictions.[37] Finally, MSCs can be used in the laboratory, for the production of induced pluripotent stem cells,[38] or as a feeding layer support for the growth of induced pluripotent stem[39] and hematopoietic stem cells (HSCs).[40,41]

Under this scope, and in view of the rapidly increasing applications of MSCs, the idea of banking controlled, ready-to-use cells appears appealing. However, for a large-scale banking of clinical-grade MSCs to be possible, the existence of an abundant cell source, easily accessible with noninvasive methods, is necessary. UC-WJ could play that role. The clear advantages of UC-WJ, compared to other sources of MSCs, are the ease of procurement of the cell source that involves no invasive procedures, the fact that UC-WJ MSCs appear to be immune privileged[42] and could, therefore, be used in an allogeneic setting, and finally their greater expansion potential.[43] Additionally, UC is routinely collected, with every cord blood unit donation, and cryopreserved in the Hellenic Cord Blood Bank, as a source of biologic backup material for testing. A piece of this UC could potentially serve for the isolation and banking of MSCs from WJ. Although recent studies have either addressed the question of the optimal MSC isolation method from the UC[44,45] or validated cryopreservation conditions for UC-WJ MSCs,[46,47] to our knowledge, there are no data combining these two critical variables for the MSC banking.

Thus, the aim of this study was to test whether freezing and storage, along with different isolation methods may affect the growth kinetics and the phenotypic and the functional characteristics of UC-WJ MSCs. The possibility of banking UC tissue rather than isolated MSCs was also envisaged. This quest is of great clinical relevance for the development of a UC-WJ MSCs bank within a cord blood bank. This MSC bank would enhance the main HSC banking activity providing with MSCs to be used for the expansion of HSCs and/or the treatment of GVHD. More importantly, the MSC bank could also allow a diversification of services, supplying with MSCs other clinical applications such as regenerative medicine or tissue engineering.

MATERIALS AND METHODS

Collection of UC

After informed consent was obtained from the mothers by experienced midwives trained in cord blood collection, fresh UCs (5 to 10 cm) were collected from normal deliveries (gestational ages, 36-40 weeks) and stored into Earl's balanced salt solution (EBSS, Gibco, Invitrogen, Paisley, UK) complemented with 100 U penicillin/1000 U streptomycin (Gibco) and processed in less than 24 hours. The collections were performed in accordance with the ethical standards of the Greek National Ethical Committee and were approved by our institution's ethical board. The UC tissues were processed according to the schematic representation presented in Fig. 1.

UC preparation and culture of isolated cells

Initial treatment of UC-WJ consisted of removing blood vessels by blunt dissection, and the remaining connective tissue (WJ) was trimmed with scissors and scalpels into 2- to 3-mm^3 pieces. Part of these tissue fragments was processed using two different protocols, while the rest of the tissue fragments were immediately frozen and processed after 1 week, 1 month, and 6 months (Fig. 1). Specifically:

Protocol 1: enzymatic isolation of UC-WJ–derived cells

Initially, UC tissue fragments were treated for 16 hours (overnight) at 37°C with 0.2% collagenase (Sigma-Aldrich, Inc., St Louis, MO) and 0.1% hyaluronidase (Sigma-Aldrich) and then treated with 0.25% trypsin-EDTA solution (Sigma-Aldrich) for 30 minutes at 37°C with agitation. After trypsin digestion, cells were washed in Earl's balanced salt solution, resuspended in culture medium, and counted. They were then divided into two equal parts. One part (approx. 3.75×10^5 cells) was directly frozen in liquid nitrogen (F). The other part was plated at a density of 5×10^3 cells/cm^2 in 75-cm^2 flasks and cultured with growth medium consisting of α-minimum essential medium (α-MEM, Gibco) supplemented with 2 mmol/L L-glutamine (Gibco), 100 U penicillin/1000 U streptomycin, and 15% fetal bovine serum (FBS, Gibco) and cultured (Primoculture I [P-I]) under standard conditions (37°C, 5% CO_2 [vol/vol] in air). Upon reaching subconfluency (approx. 20 days) cells were separated into two equal parts. One part of the cells was frozen (FP-I) for further use and the second part of P-I was processed for differentiation and immunophenotypic assays.

Protocol 2: WJ explant culture

In an alternate isolation method UC tissue fragments were placed into six-well plates (Costar, Corning Life Sciences,

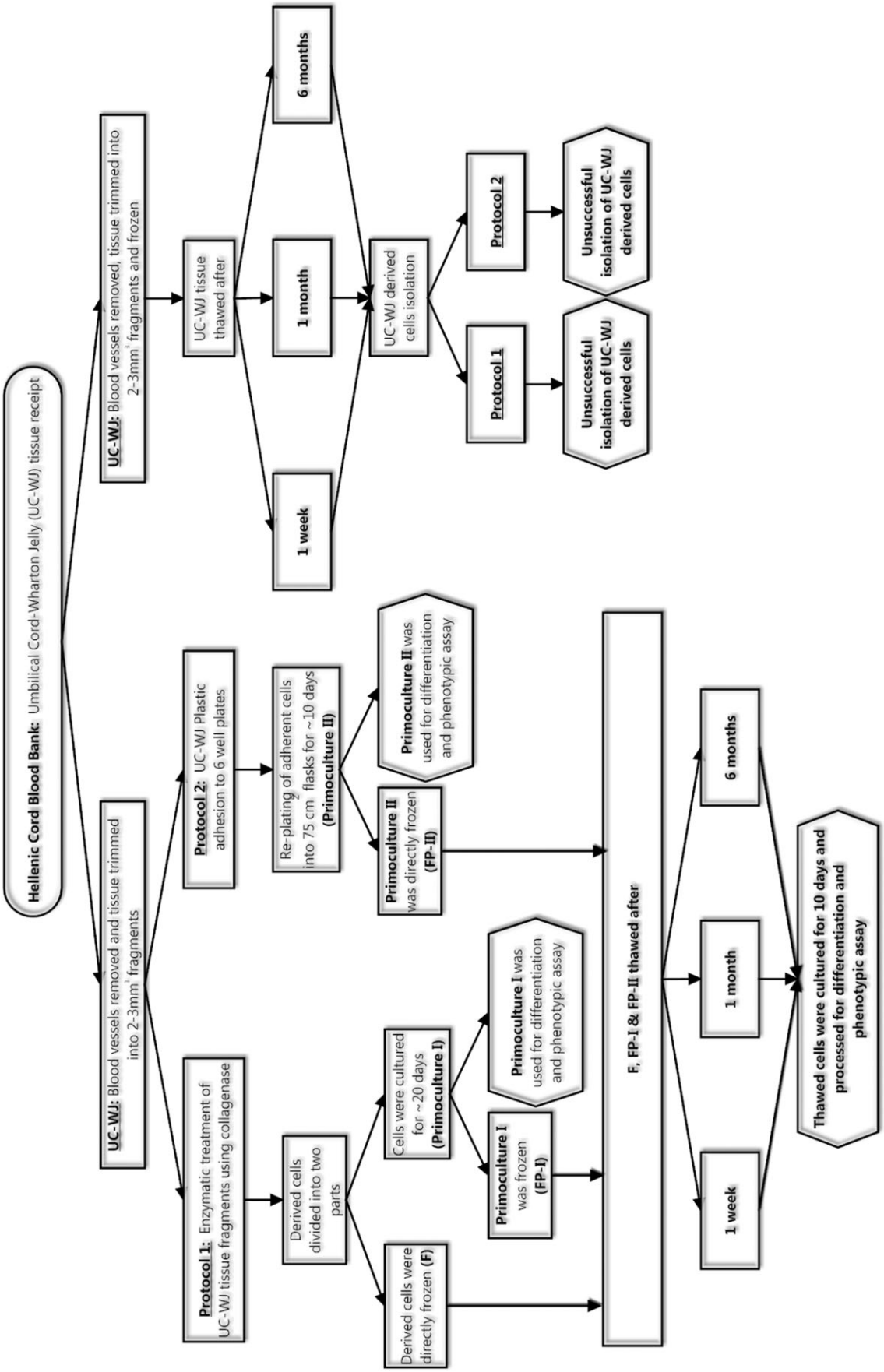

Fig. 1. Flow chart of the experimental design.

Canton, MA) and cultured with growth medium under standard conditions. Upon appearance of sufficient number of adherent cells (approx. 5 days) in the tissue culture plates, those were detached using 0.25% trypsin-EDTA solution, washed, and replated (Primoculture II [P-II]) into 75-cm^2 flasks (Costar). On reaching 80% of confluence (approx. 10 days) cells were trypsinized, washed, and resuspended in their culture medium. These UC-WJ–derived cells were also divided into two equal parts. The first part of the P-II cells was frozen (FP-II) for further use, whereas the second part was further processed for differentiation and immunophenotypic assays.

Freezing procedures

All UC-WJ–derived cells, at the end of Ps-I and -II, were placed in a precooled (4°C) cryopreservation solution consisting of 10% DMSO, 5% glycerol, and 20% FBS in α-minimum essential medium to a final concentration of 10^6 cells/mL. Moreover, 10 to 20 UC-WJ unprocessed tissue fragments of 2 to 3 mm^3 were placed in 1 mL of the same medium. Freezing of the cells initiated immediately, placing them in a container designed to gradually drop temperature by 1°C/min (Mr Frosty, Nalgene, Thermo Scientific, Waltham, MA) down to –80°C while unprocessed tissue fragments were kept at 4°C for 30 minutes before being placed in the freezing container, to allow for the cryoprotectant to penetrate the tissue. Thus, cells and tissue temperature gradually reached –80°C and then were subsequently stored in the liquid phase of liquid nitrogen at –196°C for 1 week, 1 month, and 6 months.

Cell viability counts and growth rate determination

Fresh and cryopreserved cell viability counts were determined using trypan blue staining. An automated cell counter system (Countess, Invitrogen, Paisley, UK) was used for the counts. Additionally, growth rate, for each culture, was calculated by means of cell doubling time (CDT). The equation for CDT determination was

$$CDT = \frac{\log_{10}\left(N/N_0\right)}{\log_{10}(2)} \times (T),$$

where N is the number of cells at the end of the culture, N_0 is the number of cells seeded, and T is the culture duration in hours.

Immunophenotyping analysis of MSCs

For further characterization, cell surface antigen phenotyping was performed to all cultured cells using flow cytometry. Cells were labeled with fluorescein isothiocyanate–conjugated anti-CD105 (Serotec, Oxford, UK), CD29 (Dako, Glostrup, Denmark), CD44 (Dako), CD58 (Immunotech, Beckman Coulter, Marseille, France), and CD51 (Immunotech) and with phycoerythrin-conjugated anti-CD90 (Immunotech), CD73 (Pharmingen, Becton Dickinson France S.A.S. Belgian Branch, Erembodegem, Belgium), CD34 (Immunotech), and HLA Class I (Dako) mouse anti-human monoclonal antibodies (MoAbs). Epitopes CD45 and HLA Class II were assessed with PC5-conjugated mouse anti-human MoAbs (IOTest, Immunotech). Mouse isotype antibodies served as control (Pharmingen) and 10,000 labeled cells were acquired. Stained cells were counted in a flow cytometer (Epics XL, Beckman Coulter, Marseille, France) and the results were analyzed with the software (EXPO32 ADC, Beckman Coulter).

Differentiation procedures

Differentiation capacity was assessed by attempting to differentiate UC-WJ–derived cells into the adipogenic, osteogenic, and chondrogenic lineages. Osteogenic differentiation was induced on cultured UC-WJ–derived cells using basal medium (MesenCult, StemCell Technologies, Vancouver, BC, Canada) supplemented with 15% (vol/vol) osteogenic stimulatory supplements (StemCell Technologies), 10^{-8} mol/L dexamethasone (StemCell Technologies), and 50 μg/mL ascorbic acid (StemCell Technologies). Osteogenic differentiation was assessed with Alizarin red S (Sigma-Aldrich) staining. Adipogenic differentiation was induced on cultured UC-WJ–derived cells with the use of the commercially available adipogenic differentiation kit (StemCell Technologies). Accumulation of lipid vacuoles was visualized with 0.5% (vol/vol) oil red O. Finally, for chondrogenic differentiation a micromass culture system was used. Chondrogenic medium consisted of high-glucose DMEM (Gibco) supplemented with 100 nmol/L dexamethasone (StemCell Technologies), 35 μg/mL ascorbic acid-2-phosphate (StemCell Technologies), 10 ng/mL transforming growth factor-β1 (Sigma-Aldrich), liquid medium supplement (ITS$^+$ premix, Sigma-Aldrich), and 1 mmol/L sodium pyruvate (Sigma-Aldrich). Chondrogenic differentiation was assessed with alcian blue (Fluka, Sigma-Aldrich) staining of pellet paraffin-embedded microsections.

Statistical analysis

Data are presented as mean ± SD. The Kolmogorov-Smirnov test was employed to investigate the normality of the variables distribution. If the F distribution was significant, the groups were compared by the two-sample t test (equal variance hypothesis was controlled using the Fisher or the Levene's test according to the variables distribution). A p value of less than 0.05 was considered to be significant. Computer software (Minitab Statistical

TABLE 1. Cell culture kinetics of freshly isolated and thawed UC-WJ MSCs

	Fresh cells	Thawed cells		
MSCs	P-I		FP-I	
Freezing duration		1 week	1 month	6 months
Number	10	10	10	10
Number of isolated cells ($\times 10^5$)	1.75 ± 0.94	NA	NA	NA
Mean cell viability (%)	83 ± 17	75 ± 21	81 ± 12	79 ± 16
% of cultures with cell growth (number)	100 (10)	100 (10)	100 (10)	100 (10)
CDT (hr)	141.30 ± 43.45†	123.06 ± 46.16	121.25 ± 2.40	148.63 ± 25.40
Final cell yield ($\times 10^6$)	3.42 ± 1.8	NA	NA	NA
MSCs	P-II		FP-II	
Freezing duration		1 week	1 month	6 months
Number	10	10	10	10
Number of isolated cells ($\times 10^5$)	3.02 ± 0.66	NA	NA	NA
Mean cell viability (%)	93 ± 5	71 ± 17	73 ± 25	83 ± 16
% of cultures with cell growth (number)	100 (10)	100 (10)	100 (10)	100 (10)
CDT (hr)	87.22 ± 19.28‡	134.43 ± 45.50‡	127.80 ± 26.41‡	143.41 ± 46.69‡
Final cell yield ($\times 10^6$)	4.57 ± 1.9	NA	NA	NA
MSCs			F	
Freezing duration		1 week	1 month	6 months
Number	10	10	10	10
Number of isolated cells ($\times 10^5$)	1.75 ± 0.94	NA	NA	NA
Mean cell viability (%)	83 ± 17	53 ± 19	62 ± 25	49 ± 22
% of cultures with cell growth (number)	Not cultured	10 (1)	0 (0)	10 (1)
CDT (hr)	Not cultured	209.27	NA	259.88

* Overview of the culture kinetics of fresh (P-I and P-II) and thawed (FP-I, FP-II, and F) UC-WJ MSCs. Generally, both isolation methods had comparable results as shown by P-I and P-II comparison with the exception of CDT that was calculated lower in P-II UC-WJ MSCs in respect to P-I cells. When those cells were frozen and then thawed (FP-I and FP-II) they remained equivalent for every cryopreservation period studied. Finally, comparison of postfreezing FP cells with their corresponding prefreezing P cells presented only minor differences between FP-II and P-II CDTs. In contrast, F UC-WJ MSCs showed poor results at all levels and no further statistical analysis was possible.

† $p = 0.120$.

‡ $p = 0.021$, $p = 0.028$, and $p = 0.011$ for cells cryopreserved 1 week, 1 month, and 6 months, respectively.

Software, Release 13.1, Minitab, Inc., State College, PA) was used to perform the statistical analysis.

RESULTS

Isolation of UC-WJ MSCs from UC-WJ tissue fragments

Two protocols were used for MSC isolation from both fresh and thawed UC-WJ tissue fragments (Fig. 1). The use of Protocol 1 on thawed UC-WJ tissue fragments resulted in the appearance of a few viable cells migrating on the plastic culture surface. However, these cells did not proliferate and did not have any distinctive morphologic characteristics. Similarly with Protocol 2 (enzymatic digestion), no viable cells were recovered from thawed UC-WJ fragments. In contrast, all attempts to isolate MSCs from fresh UC-WJ tissue were successful (Table 1). The number of P-II UC-WJ MSCs recovered applying Protocol 2 was $3.02 \times 10^5 \pm 0.66 \times 10^5$ cells per cm of fresh tissue (Table 1), while a mean of $1.75 \times 10^5 \pm 0.94 \times 10^5$ viable P-I UC-WJ MSCs were recovered per cm of fresh tissue applying Protocol 1. Furthermore, P-II UC-WJ MSCs grew significantly ($p = 0.120$) faster than P-I UC-WJ MSCs with mean CDTs of 87.22 ± 19.28 and 141.30 ± 43.45 hours, respectively. The final yield of P-II cells at confluence was $4.57 \times 10^6 \pm 1.90 \times 10^6$ cells after 10 days of culture, while the final population of P-I UC-WJ MSCs at confluence was $3.42 \times 10^6 \pm 1.80 \times 10^6$ after 20 days of culture (Table 1).

As a first approach toward the comparative characterization of MSCs, we analyzed the morphology of cells developed in cultures using all protocols. Thus, a characteristic spindle shape–like morphology was observed in both early P-I (Fig. 2A) and P-II UC-WJ MSCs (Fig. 2B) and confluent cultures (Figs. 2C and 2D, respectively).

Culture characteristics of cryopreserved UC-WJ MSCs

After an initial period of cryopreservation of 1 week, 1 month, and 6 months all FP-I and FP-II, as well as F-UC-WJ MSCs were retrieved from liquid nitrogen and cultured. When 83 ± 17% viable P-I UC-WJ MSCs were cryopreserved, 75 ± 21, 81 ± 12, and 79 ± 16% viable FP-I UC-WJ MSCs were recovered at thawing after 1 week, 1 month, and 6 months, respectively (Table 1), showing a good preservation of the cells' viability in liquid nitrogen. Similarly, good preservation of viability was observed when 71 ± 17, 73 ± 25, and 83 ± 16% viable FP-II UC-WJ MSCs were recovered for the same periods as mentioned, while prefreezing P-II cell viability was 93 ± 5% (Table 1). However, this was not the case for F UC-WJ MSCs; their viability dropped to 53 ± 19, 62 ± 25, and 49 ± 22% (after 1 week, 1 month, and 6 months of cryopreservation,

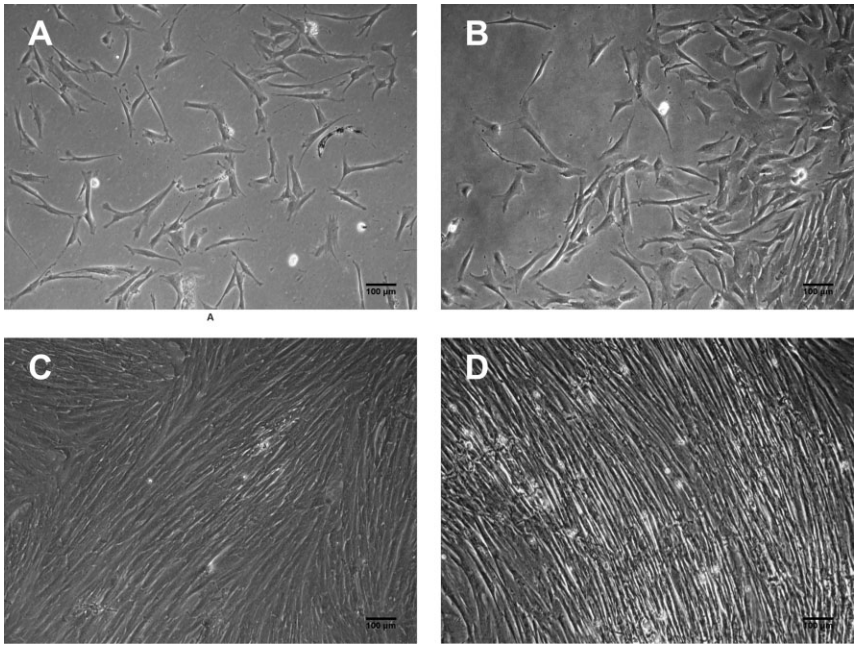

Fig. 2. Morphologic appearance of UC-WJ MSCs. The P-I (A) and P-II (B) took on the appearance of long spindle-shaped fibroblast cells and began to form colonies after the first 5 days of culture. P-I became confluent after 20 days (C) and P-II after 10 days (D), while their morphologic aspect was maintained (magnification = ×100).

respectively) even though it had been measured to be 83 ± 17% at the time of freezing (Table 1). In addition FP-I and FP-II UC-WJ MSCs were successfully cultured independently of the cryopreservation period and their CDTs were comparable to each other (Table 1). Adherent cells with a characteristic spindle shape–like morphology were already visible on the fifth day of culture of FP-I (Figs. 3A-3C) and FP-II UC-WJ MSCs (Figs. 3E-3G) cryopreserved for 1 week, 1 month, and 6 months. This morphology was maintained for the entire culture period until cells reached confluence (Figs. 3D and 3H). In contrast, of all F UC-WJ MSC cryopreserved samples, only in one case of 1 week (Fig. 3I) and that of 6 months (Fig. 3J) adherent wide cells, with irregular, rather than spindle-like shape, appeared on the 5th day of culture, but cells grew slowly and never reached confluence.

Immunophenotypic characterization

Immunophenotyping was carried out using a panel of antibodies to a number of surface antigens routinely used for the characterization of MSCs. All UC-WJ MSCs were analyzed at the end of the primoculture period, that is, on Day 20 (P-I) or Day 10 (P-II) from culture initiation. Thawed cells (FP-I and FP-II UC-WJ MSCs) were analyzed on first passage (i.e., on Day 10) after thawing. Both P-I and P-II UC-WJ–derived cell lines presented an MSC characteristic phenotype (Fig. 4). They were negative for hematopoietic markers CD34, CD45, and HLA Class II. Additionally, all UC-WJ MSC lines were found to be positive for integrins CD29 and CD51 and for matrix receptors CD44, CD58, and CD105 and positive for CD90, CD73, and HLA Class I. Freezing and thawing for all periods studied did not seem to affect the immunophenotype and all UC-WJ MSCs lines were found to retain the same expression patterns as shown by the results presented in Table 2. Direct comparison of prefreezing P-I and P-II UC-WJ MSCs with the postthawing FP-I and FP-II UC-WJ MSCs, respectively, showed some minor variability in the intensity of several markers' expression. Thus, regarding P-I UC-WJ MSCs, 91.3 ± 4.4% of the cells were positive for CD29, while 80.9 ± 2.4% of FP-I UC-WJ MSCs thawed after 1 month of cryopreservation were positive for the same marker. In contrast, CD51 expression was higher (85.5 ± 6.0% instead of 64.0 ± 7.1%) in FP-I UC-WJ MSCs thawed after 1 month of cryopreservation and remained high in cells cryopreserved for 6 months (81.6 ± 2.2%). The marker CD90 was expressed in 92.8 ± 0.2% of FP-I UC-WJ MSCs thawed at 6 months and only in 86.7 ± 4.4% of P-I MSCs. Similarly, in cells isolated with Protocol 2, CD51 expression showed greater variability between fresh P-II (95.8 ± 3.3% positive cells) and thawed FP-II UC-WJ MSCs cryopreserved for 1 week and 6 months (79.4 ± 17.4 and 84.8 ± 11.7% positive cells, respectively). Finally, CD44 expression in FP-II UC-WJ MSCs cryopreserved for 1 month was measured to be 79.8 ± 17.6%, which was significantly lower than the 96.5 ± 3.6% observed in P-II UC-WJ MSCs.

Differentiation potential of UC-WJ MSCs

The differentiation potential of MSCs isolated using the protocols described in Fig. 1 was analyzed under particular culture conditions that favor osteogenic, adipogenic, or chondrogenic differentiation. MSCs obtained at completion of P-I and P-II or FP-I and FP-II cultures were exposed to osteogenic medium for 3 weeks and their osteogenic differentiation potential was observed in their distended cell bodies, in close proximity to each other. Alizarin red S stain specific for calcium mineralization showed direct evidence of calcium deposits as amorphous accumulations in P-I (Fig. 5A) and P-II (Fig. 5D) at the end of the third week. The osteogenic differentiation potential was also maintained in FP-I (Fig. 5G) and FP-II UC-WJ MSCs (Fig. 5J) no matter the cryopreservation period as it was shown in the representative result in 5 months of

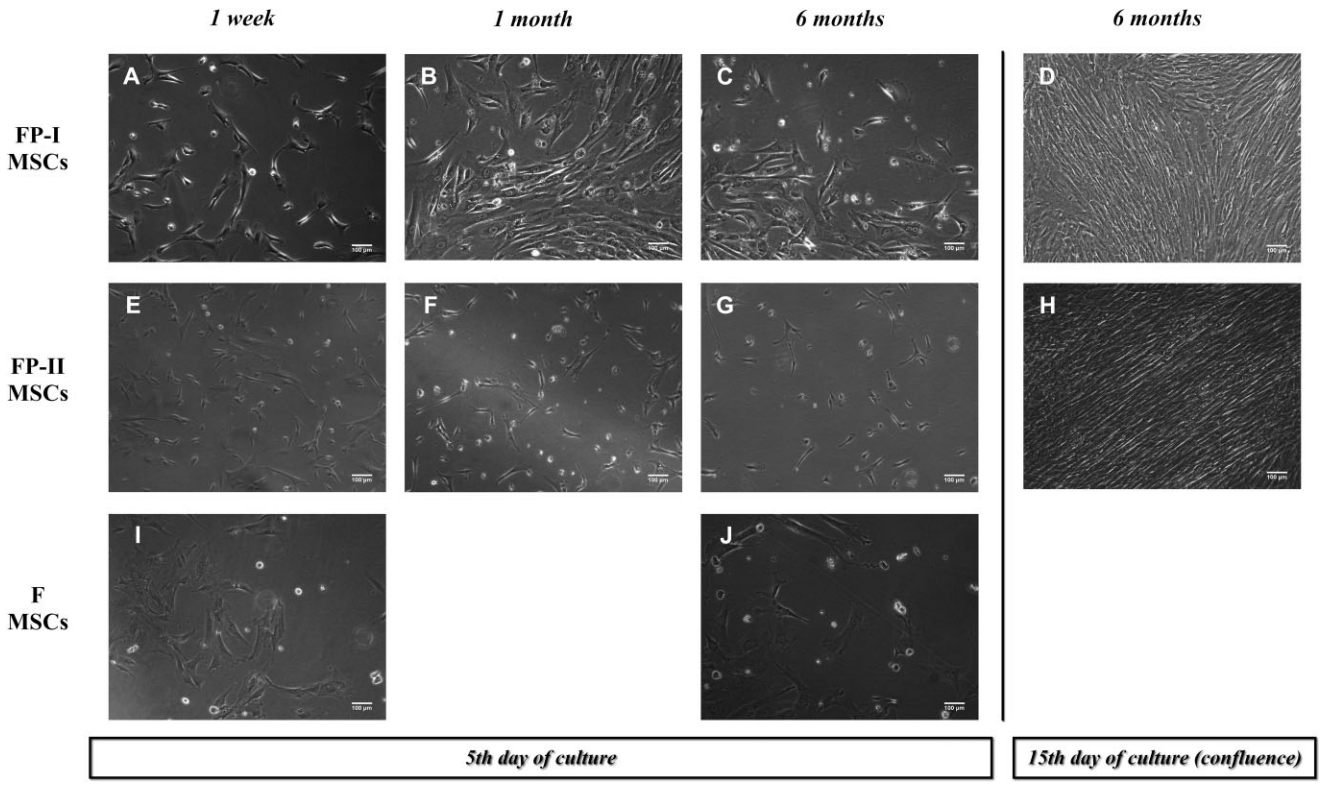

Fig. 3. Representative morphology of all cultured FP-I and FP-II UC-WJ MSCs. On the fifth day of culture, FP-I cells cryopreserved for 1 week (A), 1 month (B), and 6 months (C) as well as FP-II cells cryopreserved for the same periods (E, F, and G, respectively) had the spindle shape–like characteristic morphology of MSCs. This morphology was maintained throughout the culture period of 15 days until confluency as shown by representative pictures of FP-I (D) and FP-II (H) confluent cells (cryopreserved for 6 months). In contrast, in cultures of F UC-WJ MSC cryopreserved for 1 week (I) and 6 months (J), cells appeared wider and with an irregular shape, showing no spindle shape–like morphology, in the early stages (5 days), and they never reached confluence (magnification = ×100).

cryopreservation. On the other hand, although all cell lines were cultured for up to 21 days in adipogenic medium they did not produce a mature adipocyte phenotype. Oil red O staining revealed the presence of limited number of lipidic inclusions, in P-I (Fig. 5B), P-II (Fig. 5F), FP-I (Fig 5H), and FP-II UC-WJ MSCs (Fig. 5K).

The differentiation potential toward the chondrogenic lineage was assessed, culturing UC-WJ MSCs (P-I, P-II, FP-I, and FP-II) in a pellet micromass system. Incubation of MSC pellets in chondrogenic medium for 3 weeks yielded a matrix-rich micromass accompanied by accumulation of proteoglycans and glycosaminoglycans as evidenced by alcian blue staining (Figs. 5C, 5F, 5I, and 5L).

DISCUSSION

MSCs have been in the focus of biomedical science for the past few years. Even though details of the mechanisms implicated in their therapeutic action are not yet clarified, they are used in an increasing number of clinical trials. Since the initial isolation of MSCs from the UC tissue, WJ is being considered as a one of the most important alternatives to marrow due to the great abundance and the easy procurement of the biologic material as well as the intrinsic characteristics of UC-WJ MSCs. This study attempted to evaluate the optimal MSC isolation and cryopreservation method from UC-WJ, as a first step toward a clinical-grade MSC banking.

We compared two different methods for MSC isolation from UC-WJ. The first was based on enzymatic digestion of the connective tissue as previously described[10,11] (Protocol 1) while the other was an explant culture method (Protocol 2). Although MSC isolation was 100% successful using both methods, allowing mean recoveries of $1.75 \times 10^5 \pm 0.94 \times 10^5$ and $3.02 \times 10^5 \pm 0.66 \times 10^5$ cells/cm tissue treated, respectively, primocultures of UC-WJ MSCs isolated with the explant method (P-II) grew faster with a mean CDT of 87.22 ± 19.28 hours while mean CDT for P-I cells isolated with the enzymatic digestion method was 141.30 ± 43.45 hours. This result agrees with recently published data showing increased MSC yield and viability of explant cultures when compared to enzymatic isolation.[44]

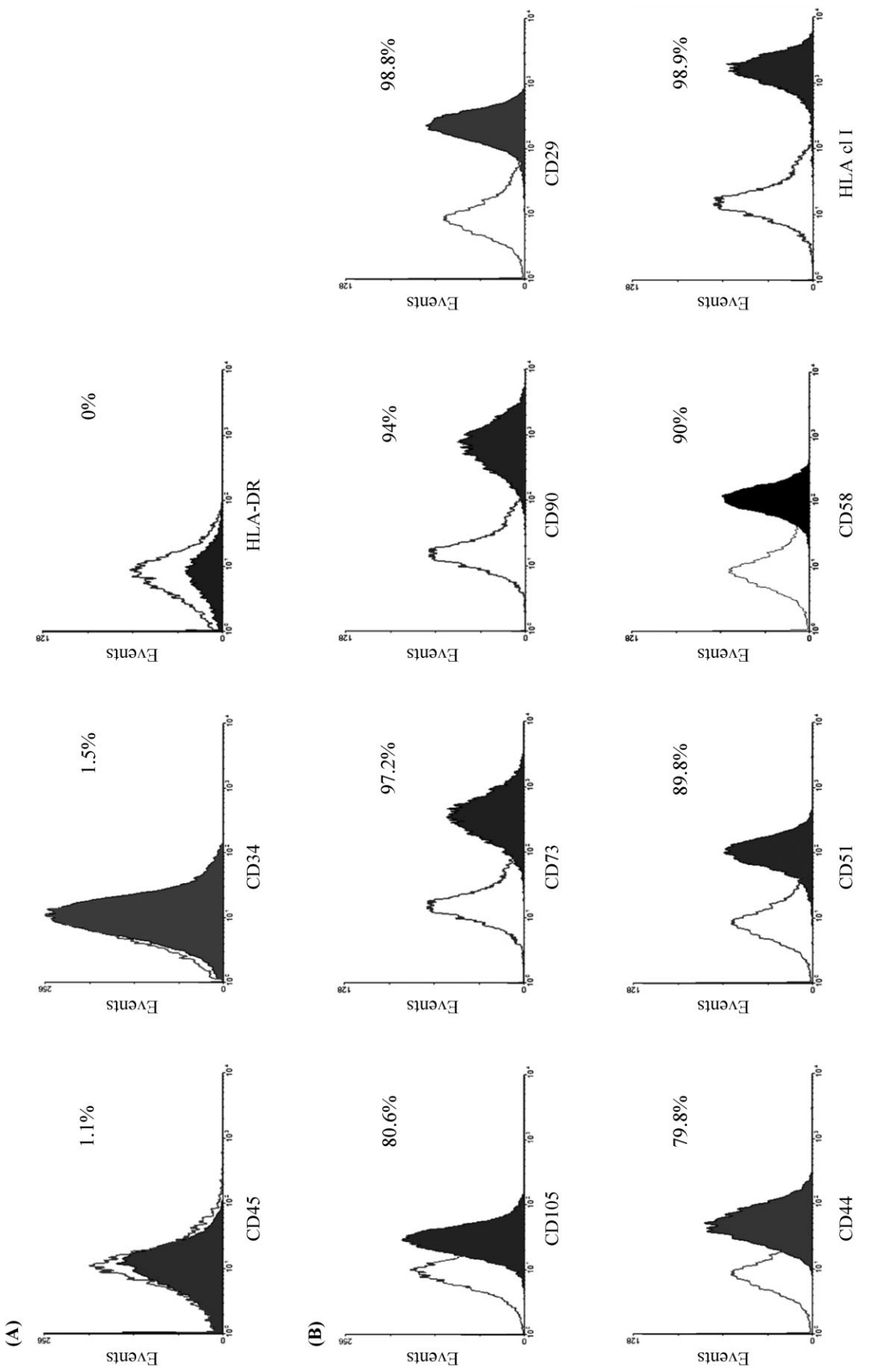

Fig. 4. Flow cytometric analysis of UC-WJ MSCs. All UC-WJ MSCs examined expressed the same immunophenotype. The surface antigens of UC-WJ MSCs were positive for CD29, CD44, CD51, CD58, CD75, CD90, CD105, and HLA Class I and negative for CD34, CD45, and HLA-DR. The graph represents FP-II UC-WJ MSCs at Passage 1 cryopreserved for 6 months. The results confirmed that the cells were of a nonhematopoietic MSC lineage. Solid lines represent isotype controls, and filled curves, specific staining.

TABLE 2. Flow cytometry analysis of UC-WJ MSCs*

	Percentage of marker expression								
			FP-I MSCs			FP-II MSCs			F MSCs
Freezing time:	P-I MSCs	P-II MSCs	1 week	1 month	6 months	1 week	1 month	6 months	1 week
CD105	80.6 ± 8.4	94.9 ± 3.3	86.8 ± 13.5	83.9 ± 17	88.4 ± 1.8	89.9 ± 9.1	92.4 ± 3.8	92.2 ± 4.2	86.7
CD90	86.7 ± 2.4	97.0 ± 2.8	90.9 ± 2.4	94.4 ± 3.7	92.8 ± 0.2† p = 0.036	95.1 ± 4.9	95.2 ± 4.9	90.9 ± 7.2	90.8
CD73	95.0 ± 1.4	91.7 ± 6.0	94.8 ± 2.6	94.7 ± 1.7	93.8 ± 0.8	91.4 ± 5.4	93.0 ± 6.4	98.0 ± 1.6	86.9
CD29	91.3 ± 4.4	97.7 ± 3.2	87.2 ± 2.5	80.9 ± 2.4† p = 0.049	87.1 ± 8.0	97.5 ± 1.0	92.0 ± 6.3	98.8 ± 1.0	92.9
CD44	79.9 ± 8.1	96.5 ± 3.6	78.7 ± 17.7	88.5 ± 4.1	91.8 ± 3.3	93.0 ± 1.8	79.8 ± 17.6† p = 0.042	94.6 ± 6.0	71.5
CD51	64.0 ± 7.1	95.8 ± 3.3	70.3 ± 13.1	85.5 ± 6.0† p = 0.041	81.6 ± 2.2† p = 0.040	79.4 ± 17.4† p = 0.037	92.8 ± 3.6	84.8 ± 11.7† p = 0.044	91.2
CD58	74.4 ± 7.6	88.0 ± 14.1	75.7 ± 16.6	81.8 ± 1.9	88.7 ± 7.3	79.0 ± 7.9	86.9 ± 8.4	90.0 ± 9.5	81.1
HLA Class I	91.3 ± 4.4	96.3 ± 5.6	87.4 ± 2.1	87.3 ± 5.2	94.3 ± 0.1	96.1 ± 3.7	93.3 ± 10.5	88.5 ± 15.4	98.1
CD34	2.2 ± 2.0	3.1 ± 3.9	1.9 ± 1.5	2.4 ± 2.0	3.6 ± 4.1	2.5 ± 2.5	1.8 ± 2.1	1.9 ± 2.4	2.1
CD45	1.4 ± 2.8	1.2 ± 0.9	1.7 ± 1.6	0.1 ± 0.5	1.6 ± 1.0	1.8 ± 1.6	0.2 ± 0.1	1.3 ± 1.8	0.3
HLA Class II	0.1 ± 0.2	0.2 ± 0.4	0.2 ± 0.2	0.3 ± 0.1	0.5 ± 0.2	0.6 ± 0.5	0.1 ± 0.0	0.3 ± 0.4	0.01

* Percentages of all UC-WJ MSCs expressing surface markers as determined by flow cytometry. The percentage of expression for each marker is indicated as the mean of all UC-WJ tissue fragments (n = 10) of each group.
† Significant at the p value specified.

The fact that MSCs were recovered at the end of explant cultures could explain the above findings, since the cells (P-II UC-WJ MSCs) were already in an exponential growth phase when Primocultures 2 initiated. In contrast, UC-WJ MSCs were put into P-I directly after an enzymatic digestion stress. Nevertheless, sufficient numbers of cells were recovered for cryopreservation using both methods ($3.42 \times 10^6 \pm 1.80 \times 10^6$ and $4.57 \times 10^6 \pm 1.90 \times 10^6$ for Isolation Methods 1 and 2, respectively). Those cells had MSC immunophenotype, expressing the characteristic surface markers CD105, CD93, CD70, CD29, CD44, CD51, CD58, and HLA Class I antigen, at a comparable level while they were negative for hematopoietic markers CD45, CD34, and HLA Class II. Both P-I and P-II MSCs were capable of at least bilinear differentiation to osteocytes and to chondrocytes but they did not undergo complete adipogenic differentiation after 21 days of induction. This observation is in accordance with previous reports that MSCs of fetal origin have diminished adipogenic differentiation potential.[48,49]

Trypan blue staining indicated that the cell viability was maintained after FP-I and FP-II UC-WJ MSCs have been thawed. For FP-I UC-WJ-MSCs, postthawing viability ranged from a mean of 75 ± 21% to a mean of 81 ± 12% depending on the cryopreservation period while the range of FP-II UC-WJ MSCs postthawing mean viability was from 71 ± 17% to 83 ± 16%. The phenotypic characteristics of thawed cells were maintained regardless of the cryopreservation period as confirmed by flow cytometric analysis.

In cases where statistical difference in the surface antigen expression was observed, before freezing and after thawing, a high SD characterized the measurements. This was true for CD51 expression, which consistently showed great variation between samples. Similarly, discrepancies linked to the measurement method could account for the significant difference observed in CD44 expression of 1-month cryopreserved FP-II UC-WJ MSCs. Other differences in the surface marker expression of individual cell groups, namely, CD29 and CD90 in FP-I UC-WJ MSCs cryopreserved for 1 and 6 months, respectively, did not appear to have any biologic significance. Finally, differentiation potential of FP-I and FP-II UC-WJ MSCs was the same as that of P-I and P-II UC-WJ MSCs since they promptly differentiated into osteocytes and chondrocytes while adipogenic differentiation was not complete after the induction period.

All attempts to isolate MSCs from fresh tissue were successful; however, this was not the case with cryopreserved tissue fragments. While other groups have reported isolating MSCs from thawed umbilical tissue,[46,50] in this study we have been unable to verify these results. Despite this discrepancy, the team of Da-Croce and colleagues[46] have mentioned morphologic changes to the frozen tissue fragments and low viability of cells recovered

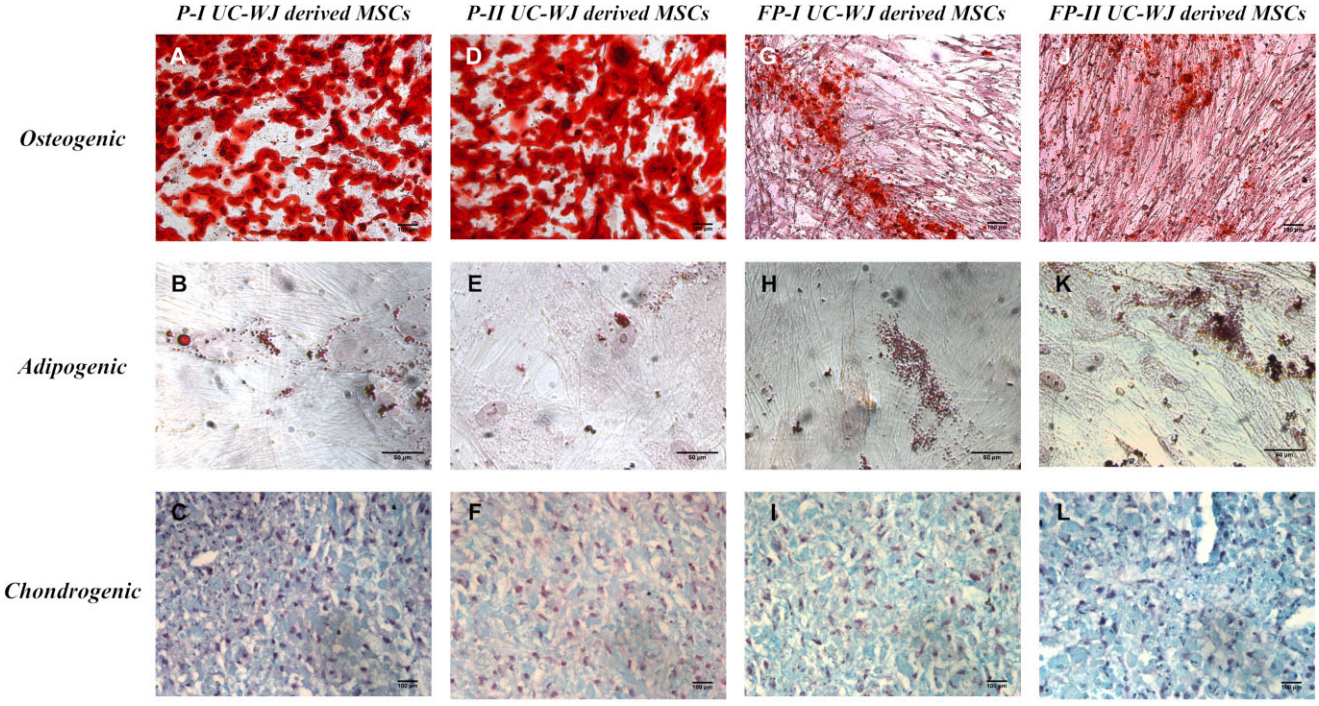

Fig. 5. Differentiation potential of fresh P-I, P-II, FP-I, and FP-II UC-WJ MSCs after 21 days of osteogenic, adipogenic, and chondrogenic induction. Calcium deposit and osteoid formation is shown by alizarin red staining in P-I (A), P-II (D), and FP-I (G) and FP-II (J) UC-WJ MSCs thawed after 6 months of cryopreservation. In contrast, there was a limited presence of lipid vesicles shown by oil red O stain of the same cells (B, E, H, and K, respectively) cultured in adipogenic favoring conditions. Finally, an alcian blue staining of the extracellular matrix in all P-I (C), P-II (F), and 6-month cryopreserved FP-I (I) and FP-II (L) UC-WJ MSCs showed the presence of proteoglycans and glycosaminoglycans in the extracellular matrix after chondrogenic induction (magnification: A, C, D, F, G, I, J, and L = ×100; B, E, H, and K = 400×).

after thawing and suggested the need for further evaluation of freezing variables such as cryoprotectant's concentration and cooling rates, variables that could account for our failure to isolate cells. Nevertheless, banking of cells instead of tissue might be of greater value for future clinical applications, since it allows storage of a fully characterized and controlled, ready-to-use, product.

Another important point that this study permitted us to clarify was the benefit of culturing the MSCs before freezing. In contrast to P-I and P-II UC-WJ–derived cells that were successfully recovered in 100% of the cases after freezing and thawing, cells cryopreserved immediately after enzymatic isolation failed to grow in culture. Only 10% of F-UC-WJ MSCs, thawed after 1 week and 6 months, and none of the stored samples thawed after 1 month of cryostorage could be recovered. Probably submitting the cells into the successive stress of prolonged enzymatic tissue digestion and freezing compromised their viability, while the primocultures allowed the MSCs to recover before cryopreservation. This hypothesis is in concordance with the low viabilities observed after thawing for the F-UC-WJ MSCs ranging from 49 ± 22% to 62 ± 25%.

Overall, this study allowed the conclusion that the optimal banking procedure for UC-WJ MSCs requires culture and expansion of the cells before cryopreservation. Although no biologic factors favor one isolation method over another, we consider that the explant technique is preferable because it is simpler and without enzymatic digestion, and therefore, causes less stress to the cells. Furthermore, the overall cost of the second isolation method is significantly lower and this is a factor that should be taken under consideration, especially for large-scale clinical banking.

Considering the rapidly increasing number of studies on MSCs from the UC that provide with a rationale for their use in a wide range of cellular therapy[30,38,51-53] or regenerative medicine[54-57] applications, banked cells might prove to be a valuable resource. We believe that our results are a first step toward the development of banking protocols for clinical-grade MSCs for novel applications. Finally, the development of MSC banks within cord blood banks could provide an important boost in stem cell banking, especially since there are indications that UC-WJ MSCs can play a significant role in CD34+ cell expansion[58] and cryopreservation[59] and GVHD incidence

reduction,[60] adding a new tool in the inventory of clinical hematology.

CONFLICT OF INTEREST

The authors have disclosed no conflicts of interest.

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