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Wharton's Jelly Mesenchymal Stromal Cells Support the Expansion of Cord Blood–derived CD34$^+$ Cells Mimicking a Hematopoietic Niche in a Direct Cell–cell Contact Culture System

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Abstract

Wharton's jelly mesenchymal stromal cells (WJ-MSCs) have been recently exploited as a feeder layer in coculture systems to expand umbilical cord blood–hematopoietic stem/progenitor cells (UCB-HSPCs). Here, we investigated the role of WJ-MSCs in supporting ex vivo UCB-HSPC expansion either when cultured in direct contact (DC) with WJ-MSCs or separated by a transwell system or in the presence of WJ-MSC–conditioned medium. We found, in short-term culture, a greater degree of expansion of UCB-CD34$^+$ cells in a DC system (15.7 $\pm$ 4.1-fold increase) with respect to the other conditions. Moreover, in DC, we evidenced two different CD34$^+$ cell populations (one floating and one adherent to WJ-MSCs) with different phenotypic and functional characteristics. Both multipotent CD34$^+$/CD38$^-$ and lineage-committed CD34$^+$/CD38$^+$ hematopoietic progenitors were expanded in a DC system. The former were significantly more represented in the adherent cell fraction than in the floating one (18.7 $\pm$ 11.2% vs. 9.7 $\pm$ 7.9% over the total CD34$^+$ cells). Short-term colony forming unit (CFU) assays showed that HSPCs adherent to the stromal layer were able to generate a higher frequency of immature colonies (CFU-granulocyte/macrophage and burst-forming unit erythroid/large colonies) with respect to the floating cells. In the attempt to identify molecules that may play a role in supporting the observed ex vivo HSPC growth, we performed secretome analyses. We found a number of proteins involved in the HSPC homing, self-renewal, and differentiation in all tested conditions. It is important to note that a set of sixteen proteins, which are only in part reported to be expressed in any hematopoietic niche, were exclusively found in the DC system secretome. In conclusion, WJ-MSCs allowed a significant ex vivo expansion of multipotent as well as committed HSPCs. This may be relevant for future clinical applications.

Keywords

hematopoietic stem and progenitor cell expansion, Wharton's jelly mesenchymal stromal cells, hematopoietic niche, secretome, extracellular matrix

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Introduction

Unrelated umbilical cord blood (UCB) transplantation has become one of the therapeutic options for pediatric patients with hematological malignancies and various genetic disorders[1]. The easy availability, the higher tolerance to human leukocyte antigen (HLA)-mismatch, and the lower incidence of graft-versus-host disease (GvHD) make UCB an elective source of hematopoietic stem/progenitor cells (HSPCs) for cell transplantation compared to bone marrow (BM) and mobilized peripheral blood counterparts[2]. More importantly, UCB-HSPCs have a higher frequency of progenitors with greater clonogenic potential compared to adult cells[3]. Nonetheless, the number of HSPCs derived from 1 UCB unit is often not sufficient for transplantation in adult patients. In this regard, several attempts have been made to expand HSPCs by using specific culture media, supplemented with different combinations of cytokines and chemical compounds, and also by coculturing them with stromal feeder layers[4]. The long-term fate of transplanted HSPCs greatly depends on their ability to adhere to the niche, with subsequent regulation of self-renewal *versus* differentiation states[5]. All of these functions are regulated by cues provided in vivo by the hematopoietic niche cellular microenvironment. The niche of BM has been studied in both human and animal models[6,7]. Specialized cell types, such as mesenchymal stromal cells (MSCs), endosteal and vascular cells, and also pericytes, comprise the BM niche. Extracellular matrix molecules are also key to maintain the balance between HSPC self-renewal and differentiation[8]. Therefore, the development of in vitro systems that could mimic the microenvironment of a hematopoietic niche would improve the ex vivo HSPC expansion strategies. Recently, numerous studies have reported that MSCs from adult and perinatal sources can be used as feeder layers to expand and maintain the undifferentiated state of HSPCs[9,10]. Nevertheless, the cellular and molecular mechanisms mediating these interactions are not fully elucidated. To date, few studies investigated how the MSCs from different sources, influenced the quantity and quality of expanded UCB-derived HSPCs in various coculture systems. Furthermore, studies are lacking with respect to a direct side-by-side comparison between cell–cell contact, noncontact, and expansion in standardized media, using UCB-derived HSPCs and MSCs. Wharton's jelly (WJ) is an attractive source of MSCs. It originates from the extraembryonic mesoderm (EM) that constitutes the mesenchymal layer surrounding the amniotic cavity and yolk sac as well as the stroma of umbilical cord (UC) and placenta[11]. EM has been shown to support embryonic and fetal hemopoietic niches[12]. The WJ-derived MSCs (WJ-MSCs), isolated from UC matrix, may be an ideal candidate for creating an effective stromal feeder layer in coculture systems. These cells can be easily harvested and readily expanded to reach a confluent monolayer in a short time[13]. Furthermore, WJ-MSCs produce several cytokines involved in the regulation of hematopoiesis, similarly to that observed in BM-MSCs. Key examples are interleukin-6 (IL-6) stem cell factor (SCF), Fms-related-tyrosine kinase-3 (Flt-3) ligand, and growth factors such as macrophage colony–stimulating factor (M-CSF), granulocyte colony–stimulating factor (G-CSF), and granulocyte/macrophage (GM) colony–stimulating factor (GM-CSF)[14,15]. It is important to note that the developmental history of WJ-MSCs further justifies a role for these cells in supporting hematopoiesis by establishing a functional niche-like environment for UCB-HSPCs. Indeed, WJ is part of the extraembryonic mesoderm, a tissue that arises early in the embryo lining and is the very first hematopoietic niche (during the vitelline primitive hematopoiesis occurring in the wall of the yolk sac)[16]. Further ahead in human development, umbilical vessels and placenta have also been proposed as sites of hematopoiesis and storage of embryonic definitive $CD34^+$ cells; this led to the concept of extraembryonic niches[17]. In this work, we investigated the role of WJ-MSCs in supporting ex vivo UCB-$CD34^+$ cell expansion. Cocultures were performed with $CD34^+$ cells cultured either directly on a WJ-MSC layer or in the presence of WJ-derived conditioned medium (WJ-CM) or in a transwell system (TS). Phenotypical and functional characterization of the cultured $CD34^+$ cells as well as systematical analysis of secretomes were performed in order to assess the role of this stromal cell population in the ex vivo expansion of HSPCs.

Materials and Methods

Isolation of WJ-MSCs

UCs (n = 12) were obtained at the Villa Sofia-Cervello Hospital, Palermo, Italy, after full-term births with written informed consent from mothers and treated in accordance with the tenets of the Declaration of Helsinki and local ethical regulations.

The UC was obtained at birth, after vaginal delivery or cesarean section, and stored in cold Hank's balanced salt solution (HBSS) (Sigma-Aldrich, Milan, Italy), supplemented with 1× of antibiotic/antimycotic (penicillin/streptomycin, PAA Laboratories, Pasching, Austria). UCs were processed for cell isolation within 12 h from partum. The isolation procedure was performed as reported previously[18]. Briefly, the collected UCs were cut into small pieces (of about 1 to 1.5 cm length) and sectioned longitudinally to expose the inner matrix, WJ, to the plastic surface of 6-well plates (6-well tissue-culture treated, CytOne). Each piece was completely covered with standard growth medium composed of Dulbecco's modified Eagle's medium (DMEM) low glucose (Gibco, Milan, Italy) supplemented with 10% heat inactivated fetal calf serum (FCS) (Gibco), 1× nonessential aminoacids (Sigma-Aldrich), and 1× penicillin/streptomycin (PAA Laboratories). The cord fragments were left in the culture medium for 15 d, with medium change every 2 d. After 15 d of culture, the cord fragments were removed from the wells, and cells attached to the plastic surface were cultured until they reached confluence. The subsequently passaged cells were collected by enzymatic treatment with 1X TrypLE Select (Gibco) and replated at 10,000 cells/cm^2.

Phenotypical Characterization of WJ-MSCs

WJ-MSCs were routinely phenotyped at the first and fourth passage by flow cytometry (FC-500 Beckman Coulter) according to the manufacturer's instructions. The antibodies used were CD90-PC5, CD105-PC7, CD34-CD45-ECD (R Phycoerythrin-Texas Red-X), HLA-DR-PE (Phycoerythrin), all supplied by Beckman Coulter, and CD73-FITC, supplied by BD Biosciences. Consistently, WJ-MSCs expressed the expected mesenchymal markers CD73, CD90, and CD105 and were negative for the hematopoietic markers CD34, CD45, and HLA-DR (data not shown). The ability of WJ-MSCs to differentiate toward osteogenic, adipogenic, and chondrogenic phenotypes was routinely assessed during culture passages as previously described[19] (data not shown).

UCB-CD34$^+$ Cell Immunoselection and Subtyping

UCB samples were collected at birth in isotonic saline solution supplemented with anticoagulant acid citrate dextrose A. CD34$^+$ cells were immunoselected using a CliniMACS instrument (CD34 microbeads kit human, CliniMacs, Miltenyi Biotec) from the mononuclear cell fraction collected by Ficoll-Paque Plus density 1.077 gradient (GE Healthcare, Milan, Italy).

The absolute numbers and the frequencies of CD34$^+$ cells were measured in UCB samples at the time 0 and after 5 d of culture by flow cytometry analysis according to ISHAGE (International Society of Hematotherapy and Graft Engineering) protocol with a single platform method (Stem Kit, Beckman Coulter, Milan, Italy) that identifies the dual-positive CD45$^+$/CD34$^+$ cell population. To exclude dead cells from the analysis, 7-aminoactinomycin D (7-AAD) was used. The cells were cryopreserved in CS110 medium (STEMCELL Technologies) until the next use.

Cell subtyping was performed by flow cytometry with a 4-color platform according to the manufacturer's instructions. Cells were stained with the following monoclonal antibodies or with the corresponding isotype controls: fluorescein isothiocyanate (FITC)-conjugated CD45, ECD-conjugated CD34, and PC5-conjugated CD38, supplied by Beckman Coulter, and PE-conjugated CD133 (Miltenyi Biotech). Data were analyzed using the Cxp Cytometer analysis software and Kaluza software (Beckman Coulter).

Coculture of WJ-MSCs and UCB-derived CD34$^+$ cells

All of the experiments were carried out in allogeneic systems. 5×10^4 WJ-MSCs at the fourth passage were seeded in each well of a 12-well plate (Costar, Corning) to reach a cell confluence of 70% to 80%. Cell growth was arrested with 20 μg/mL mytomicin C (Sigma-Aldrich) for 2.5 h at 37 °C in the presence of 5% CO_2. After incubation, the WJ-MSCs were washed twice with phosphate buffered saline (PBS) and cultured in standard growth medium for 24 h before coculturing with CD34$^+$ cells.

In all culture conditions, 2.5×10^4 CD34$^+$ cells/mL/well were grown in an animal component-free (ACF) medium (Stem Span™ ACF STEMCELL Technologies, Inc.) formulated for hematopoietic cell expansion, supplemented with a 1× cytokine cocktail (Stem Span™ CC110, STEMCELL Technologies, Inc.), and contained recombinant stem cell factor (SCF), Fms-related tyrosine kinase-3 ligand (Flt-3) and thrombopoietin (TPO). In particular, CD34$^+$ cells were directly seeded on a WJ-MSC layer (DC system) or separated from the stromal feeders by a transwell permeable support (TS; 0,4 μm Polyester Membrane 12 mm Insert, 12-well Plate Tissue Culture Treated, Costar Corning) or in the presence of WJ-CM or in ACF medium alone (expansion medium [EM]). The evaluation of the CD34$^+$ cell expansion was reported as fold increase after 5 d of culture with respect to time 0.

WJ-CM Preparation

WJ-MSCs at the fourth passage were used to produce a conditioned medium. Cells were grown in ACF medium until reaching a confluency of 70% to 80% and arrested with 20 μg/mL mitomycin C for 2.5 h at 37 °C in a 5% CO_2 atmosphere. After cytostatic treatment, cells were washed twice with PBS and incubated for 48 h in ACF medium, supplemented with a CC110 cytokine cocktail. The medium was collected and centrifuged at 1,200 rpm for 8 min to remove the cellular debris and stored at −80 °C for the subsequent analyses.

Immunocytochemical (ICC) Analysis

After culturing, cells grown in chamber slides were washed with PBS and fixed in methanol for 20 min at −20 °C. Air-dried slides were then stored at −20 °C until use. For the ICC procedure, cells were permeabilized with 0.1% Triton X-100 in PBS (Sigma-Aldrich). After a subsequent rinse with PBS, immunocytochemistry (ICC) was performed using the ICC/IHC "Histostain Plus 3rd Gen IHC Detection" kit (Invitrogen). The primary antibodies used for this study were diluted with saponin 0.1% in PBS 1× and they were Fibronectin (Sigma-Aldrich), CD34 (Dako, Milan, Italy), and Vimentin (Dako). Nuclear counterstaining was obtained using hematoxylin (Dako).

Colony Forming Unit (CFU) Assay

The ability of CD34$^+$ cells to form colonies in short-term assay after 5 d of culture in WJ-CM EM and DC conditions was assessed: 1.5×10^3 CD34$^+$ cells were plated in triplicate in the complete methylcellulose medium (MethoCult H4434 Classic, STEMCELL Technologies, Inc.) in 35-mm culture dishes and incubated at 37 °C in 5% CO_2 for 14 d.

Colonies were counted and analyzed for morphology using an inverted microscope and a scoring grid. Colony morphologies were classified as CFU-GM, burst-forming

unit erythroid (BFU-E), colony forming unit granulocytes (CFU-G), colony forming unit monocytes, and colony forming unit granulocyte/erythroid/macrophage/megakaryocytes (CFU-GEMM).

Statistical Analysis

All statistical analyses were done using GraphPad Prism (version7.02; https://www.graphpad.com/scientific-software/prism/). Results are presented as mean ± standard deviation (SD). To test statistical significance, a paired parametric *t*-test was used. Results were considered significant for P value <0.05.

Sample Preparation for Secretome Analyses

All media from different culture conditions were harvested, centrifuged (1,100 rpm at 4 °C for 5 min) and filtered through a 0.22-μm filter (Millipore) to remove cells and debris. The supernatants were dialyzed to remove and minimize charged ions and salts for the subsequent mass spectrometry (MS). After the dialysis, the samples were lyophilized and resuspended in ultrapure water in order to obtain a 100-fold protein concentration.

In Solution Digestion

The digestion step was performed adding Trypsin/Lys-C Mix (Promega) to 50 μg of proteins at a 25:1 protein:protease ratio (w/w), following the manufacturer's instructions. Peptides, obtained after the digestion, were concentrated and desalted with Pierce C18 Spin Columns (Thermo Scientific). Then the samples were lyophilized and suspended in an appropriate buffer (0.1% formic acid) for subsequent applications.

High-performance Liquid Chromatography (HPLC) and Electrospray MS

Reversed-phase HPLC separation and online mass spectrometry detection (HPLC-MS/MS) were performed using an UltiMate 3000 System (Dionex) combined with Linear Trap Quadrupole (LTQ) Orbitrap XL™ Hybrid Ion Trap-orbitrap Mass Spectrometer (Thermo Fisher, Milan, Italy) via electrospray ionization (ESI) source. The chromatographic separation was carried out on a Hypersil Gold C18 column (15 cm × 2.1 mm, 1.9 μm). Sequential elution of peptides was accomplished using a flow rate of 50 μL min^{-1} and a linear gradient from 98% solution A (0.1% formic acid) to 98% of solution B (80% acetonitrile, 0.08% formic acid) in 120 min. The gradient elution program was as follows: 2 min 98:2, 4 min 87:13; 95 min gradient from 87:13 to 50:50, 104 min 2:98, 109 min 2:98, 110 min 98:2, and 120 min 98:2 (A:B, v/v). The column temperature was set at 25 °C, and the sample tray temperature was maintained at 4 °C. For all experiments, a sample volume of 4 μL was loaded. For MS detection, the ESI source was operated in the positive mode and run with Xcalibur version 2.0 software (Thermo Fisher). High-purity nitrogen was used as the sheath, and the auxiliary gas and high-purity helium was used as the collision gas.

Data Analysis

The raw data were processed using Proteome Discoverer version 1.4 (Thermo Scientific). MS/MS spectra were sequentially searched with SequestHT and MS Amanda, both set against Homo sapiens database using the following parameters: full trypsin digest with maximum 2 missed cleavages; fixed modification carbamidomethylation of cysteine (+57.021 Da); variable modification oxidation of methionine (+15.995 Da); and phosphorylation of tyrosine, serine, and threonine (+79.966 Da). Precursor mass tolerance was 10 ppm and fragment ion tolerance was 0.8 Da. Peptide spectral matches were validated using percolator.

In Silico Analysis

In order to confirm the identified proteins, in silico analyses were carried out using the online ExPASy Proteomics Server (http://www.expasy.org) and the UniProt database (http://www.uniprot.org). For functional analysis of the proteomics data, the Database for Annotation, Visualization, and Integrated Discovery (version 6.8, https://david.ncifcrf.gov/) was applied.

Results

Phenotypic Characterization of WJ-MSCs and UCB-$CD34^+$ Cells

The MSC population derived from WJ was isolated by its ability to migrate and adhere to a plastic surface. Cells were subcultured for 4 passages prior to being used as feeders in coculture system. The morphological and phenotypical analyses, as well as the differentiative properties, were evaluated at the first and fourth passage. During culture, WJ-MSCs maintained the typical fibroblastoid morphology, with cell–cell contacts highlighted by processes extending between adjacent cells.

$CD34^+$ cells from 12 UCB independent samples were enriched for $CD34^+$ cells by immune selection to reach a purity of 90.2 ± 3.7% with a recovery of 65 ± 5%. The selected $CD34^+$ cell population was subtyped for the presence of 2 surface markers, CD133 and CD38, that identify more primitive and committed hematopoietic progenitor cells, respectively. The frequency of $CD34^+/CD133^+$ cells in the uncultured cell population was 90.2 ± 5.8, and the frequency of $CD34^+/CD38^-$ cells was 40 ± 11%.

Phenotypic, functional, and proteomic analyses were performed after 5 d of WJ-MSC/$CD34^+$ cell coculture; in our previous experiences, at this relatively short time of culture, the frequency of $CD34^+$ cells within the total expanded cell population is only slightly decreased.

UCB-CD34$^+$ Cell Expansion in Different Culture Systems

To investigate the role of WJ-MSCs to support in vitro expansion of HSPCs, the UCB-CD34$^+$ cells were cultured either directly on WJ-MSC feeders (DC condition) or separated from feeder cells by TS, or in presence of conditioned medium derived from WJ-MSCs (WJ-CM), as well as in a standard EM alone. First, we assessed that 2.5×10^4 cells/mL was the optimal seeding concentration to favor the cell expansion kinetic and that the presence of the 3 cytokines (SCF, TPO, and flt-3) known to support hematopoietic stem and progenitor cells was essential for cell growth in all tested culture conditions (data not shown). After 5 d of culture, the number of CD34$^+$ cells significantly increased in all conditions with respect to time 0 ($P < 0.0001$); in particular, we observed a 15.7 $\pm$ 4.1-fold increase in the DC system, 9.5 $\pm$ 3.3 in WJ-CM, 8.4 $\pm$ 3.3 in EM, and 5 $\pm$ 2.2 in TS (Fig. 1). Notably, the DC system resulted in a superior expansion with respect to all the other culture conditions ($P \leq 0.001$). At this time of culture, the frequency of CD34$^+$ cells over the total hematopoietic cell population decreased by about 25% to 35% in the different culture conditions with respect to the uncultured samples (Fig. 1). Analogue results have already been reported in culture systems and expansion media very close to the conditions that we used in our experiments[20]. This result shows that a portion of CD34$^+$ cells are expanded while another portion of them differentiates.

The low expansion degree observed in TS was probably influenced by a mechanical hindrance of TS per se. Preliminary experiments (not shown) indicated that the TS is able to expand CD34$^+$ cells only at very low concentrations, different from those used for this study.

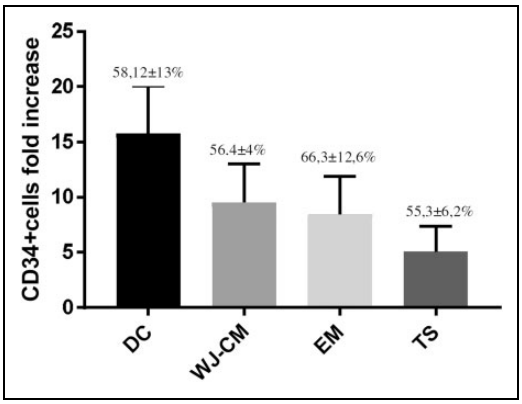

Fig. 1. The fold increase of expanded umbilical cord blood–CD34$^+$ cells after 5 d of culture in different conditions: direct contact (DC) system, Wharton's jelly mesenchymal stromal cells–conditioned medium (WJ-CM), expansion medium (EM), and transwell system (TS). This graph also reports the frequency of CD34$^+$ cells after 5 d of culture in all conditions. All results are presented as mean $\pm$ SD.

In Vitro Interactions between CD34$^+$ Cells and WJ-MSCs in the DC System

By phase-contrast microscopy, we observed within the direct coculture system that the expanded CD34$^+$ cells localized in 2 distinct compartments: a floating cell population and another one adherent to the WJ-MSCs. Figure 2 shows that the majority of hematopoietic cells are floating in the medium. These cells can be easily harvested by medium aspiration. About one-third (36.9 $\pm$ 7.1% of the total expanded CD34$^+$ cells in the DC condition) of the HSPCs are tightly bound to the stromal feeder cell layer. These adherent cells were harvested only after enzymatic treatment that breaks cell–cell or cell–matrix interactions. In fact as shown in Fig. 2, the adherent cells were either directly associated with WJ-MSCs or with the extracellular matrix (ECM) deposited by feeder cells. This is the first time that such an observation was made in a coculture system with WJ-MSCs as a feeder layer for UCB-HSPCs.

Immunocytochemistry analysis was performed to demonstrate the expression and localization of different molecules that mark specifically hematopoietic or stromal

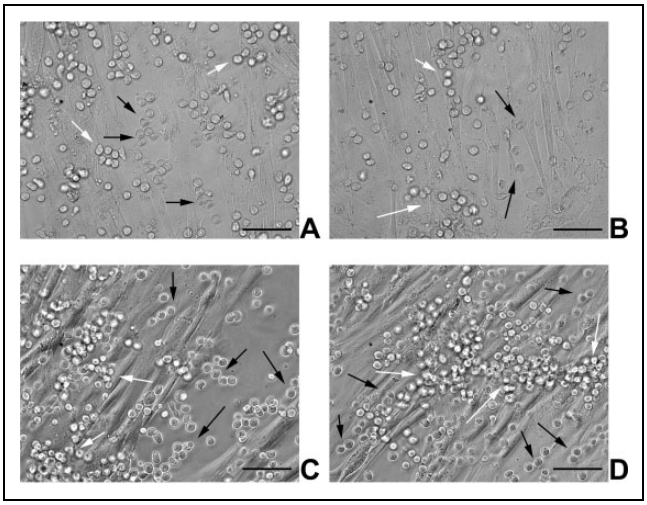

Fig. 2. Microscopical demonstration of the multiple ways of interactions between Wharton's jelly mesenchymal stromal cells (WJ-MSCs) and CD34$^+$ cells in the direct contact system. CD34$^+$ cells seeded on a layer of WJ-MSCs and cultured for 5 d expand their numbers and establish different interactions with feeder cells (A–D). Phase-contrast microscopy allows for detection of a floating population that constitutes about two-thirds of the total expanded cells (indicated by white arrows in all panels). An adherent population is represented by cells which adhere to the feeder cells or to the culture surface (indicated by black arrows in all panels). Magnification: 200×. Bar: 100 μm.

cells (Fig. 3). CD34 antibody was used to stain the HSPCs, showing the presence of the CD34$^+$ cells over the WJ-MSCs that were negative for this marker (Fig. 3A). Vimentin, a typical cytoskeletal marker of MSCs, was localized in both HSPCs and WJ-MSCs (Fig. 3B and C), confirming literature data from our group and others[18,21] HSPCs showed pseudopodia-like protrusions indicating an active migratory activity over the culture surface. (Fig. 3D), a

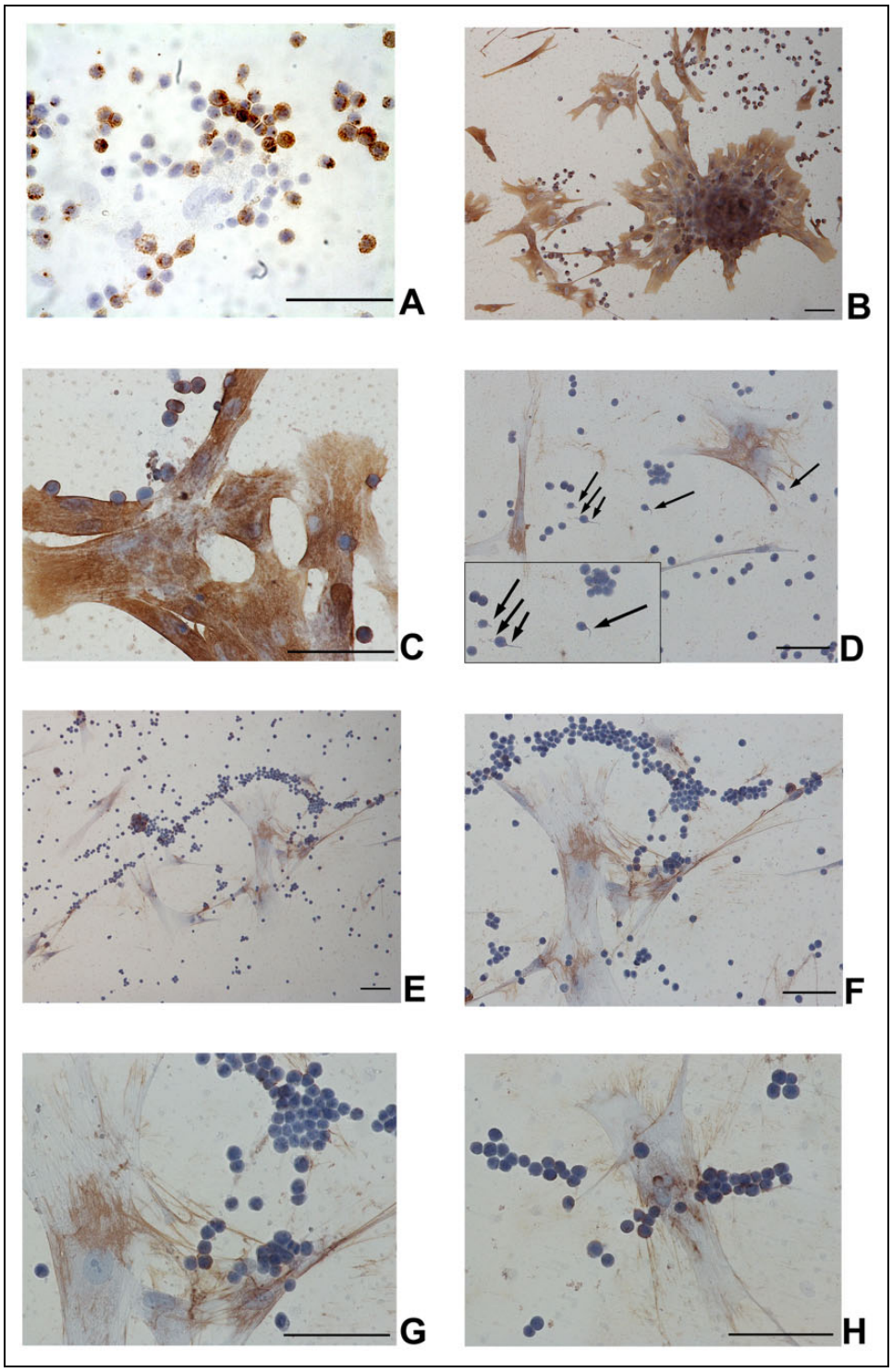

Fig. 3. Microscopic demonstration of the expression of intracellular and extracellular molecules in the direct contact (DC) system. $CD34^+$ cells belonging to the adherent fraction can be easily visualized in areas around Wharton's jelly mesenchymal stromal cells (WJ-MSCs) as shown by their staining for anti-CD34 antibody (A). (B, C) Vimentin stains both WJ-MSCs and hematopoietic stem/progenitor cells (HSPCs). Higher magnification images (400×, C) show the close association between the WJ-MSC feeder layer and HSPCs. Apart from interacting with WJ-MSCs, HSPCs clearly show arrangements directed by the extracellular fibronectin polymerized by WJ-MSCs. (D) Fibronectin antibody shows cytoplasmic stain in WJ-MSCs but not in HSPCs. The protein is polymerized in an extracellular network with fibers localized both at the periphery of cells and on the culture surface. As indicated, HSPCs show pseudopodia-like protrusions (arrows) indicating a migratory activity over the vessel surface, which is covered with fibronectin filaments. The inset shows a higher magnification view of the cellular protrusions. (E) Extracellular fibronectin directs the adhesion of HSPCs (100×). (F) Higher magnification of the panel E (200×) showing nests of HSPCs localizing across fibronectin extracellular fibers and at the periphery of WJ-MSCs. (G) High-power view (400×) of panel F showing the interactions between HSPCs and the surrounding fibronectin-rich microenvironment. (H) The peripheral positive fibronectin stain in some HSPCs (with absence of cytoplasmic stain) suggesting a close receptor-mediated interaction. Magnifications: 100× (B, E), 200× (D, F), and 400× (A, C, G, H). Bar: 100 μm.

Table 1. Frequency of Primitive $CD34^+$ Subtypes over the Total $CD34^+$ Cells.

		DC System				
	Uncultured Cells	Floating Cells	Adherent Cells	WJ-CM	TS	EM
$CD34^+/CD38^-$, %	40.2 ± 9.2	9.7 ± 7.9	18.7 ± 11.2	52.9 ± 9.0	81.9 ± 5.8	89.2 ± 4.8
$CD34^+/CD133^+$, %	90.2 ± 5.8	84.4 ± 6.6	84.0 ± 4.8	82.9 ± 8.5	79.9 ± 9.2	79.9 ± 8.1

Note: Results are reported as mean ± SD. DC = direct contact; WJ-CM = Wharton jelly–conditioned medium; TS = transwell system; EM = expansion medium.

Table 2. Frequency of Colonies Generated from $CD34^+$ Cells Cultured in Direct Contact System.

	DC System		
Colonies	Floating Cells	Adherent Cells	*P* Values
% CFU-GM + BFU-E/total colonies	35.0 ± 14	46.8 ± 13.9	0.007
% CFU-GEMM/total colonies	0.5 ± 0.5	3.5 ± 1.3	0.0001
% BFU-E(L)/total BFU-E colonies	19.0 ± 6.3	37.0 ± 14.7	0.013

Note: Results are reported as mean ± SD. DC = direct contact; CFU-GM = colony forming units–granulocyte/macrophage; BFU-E = burst-forming units–erythroid; BFU-E(L) = intermediate/large burst-forming units–erythroid; CFU-GEMM = colony forming units–granulocyte/erythroid/macrophage/megakaryocytes.

datum already reported in a similar coculture system where $CD34^+$ cells were cocultured with WJ-MSCs[20]. Fibronectin was expressed exclusively by WJ-MSCs (Fig. 3D-H). The cells showed an intense cytoplasmic staining according to their ability to produce and polymerize extracellular matrix molecules. Interestingly, fibronectin was localized extracellularly both at the periphery of WJ-MSCs and over the glass culture surface. This provided means for $CD34^+$ cells to attach firmly to both WJ-MSCs and the culture slide. This is particularly evident in Fig. 3E-G showing that the arrangement of $CD34^+$ cells follows the direction of the extracellular fibronectin fibers. High-power field images (Fig. 3H) suggest a close association between extracellular fibronectin and $CD34^+$ cells, giving the impression of a positive staining at the membrane of the cells, which is however not matched by the expected cytoplasmic stain of cells actively synthesizing the molecule. Therefore, this suggests that $CD34^+$ cells closely interact with fibronectin via receptor-mediated interaction.

Subtyping of Expanded $CD34^+$ Cells

The frequencies of $CD34^+/CD133^+$ and $CD34^+/CD38^-$ subpopulations that identify early progenitor cells were measured in all culture conditions and within the 2 hematopoietic cell fractions of the DC system. After 5 d of culture, the frequency of $CD34^+/CD133^+$ progenitor cells was slightly decreased compared to the uncultured samples in all conditions and also within the 2 cell populations of the DC system ($P \leq 0.0377$; Table 1).

Differences in the coexpression of the CD38 marker, which discriminate committed ($CD34^+/CD38^+$) from multipotent ($CD34^+/CD38^-$) progenitors[22], were observed between $CD34^+$ cells grown in direct contact with WJ-MSCs and all the other conditions. In particular, the frequency of the $CD34^+/CD38^-$ subpopulation over the total $CD34^+$ cells was significantly increased in both EM and TS with respect to that of uncultured UCB-$CD34^+$ cells. In particular, we observed a 2-fold increase in both EM and TS ($P < 0.0001$) and a 1.3-fold in WJ-CM ($P = 0.0074$; Table 1). Conversely, in the DC system, the frequency of multipotent progenitor cells decreased in both floating and adherent compartments when compared to the uncultured cells ($P \leq 0.0003$). This decline was observed to a lesser extent in the adherent cell fraction (2-fold decrease) than in the floating cells (Table 1). These results highlighted that the cell–cell contact greatly favored the production of committed hematopoietic progenitors with respect to the other tested culture conditions. Notably, in the DC condition, the $CD34^+$ cells adherent to the stromal layer comprised a higher percentage of early primitive cells when compared to the respective floating compartment (18.7 ± 11.2% vs. 9.7 ± 7.9%, $P < 0.0001$). In terms of absolute numbers, the $CD34^+/CD38^-$ early progenitors were found expanded in the DC culture system after 5 d; in particular, we observed a 2-fold increase in the floating cell population and a 3-fold increase in the adherent one (data not shown).

Overall, these data highlighted that cell–cell contact promoted both proliferation and differentiation of HSPCs resembling the main features of a hematopoietic niche. Similar results have been reported by Jing and colleagues who performed cocultures of mobilized peripheral blood-derived $CD34^+$ cells over a feeder layer of BM-MSCs. The authors suggested that the hematopoietic cells beneath MSCs featured the low proliferation rate and displayed the markers of an immature phenotype[23].

Table 3. Hematopoiesis-regulating Molecules Identified in the Media of Different Culture Conditions.

Molecules	Accession Number	Culture Condition	Role in Hematopoiesis	Hematopoietic niche	References
Secreted molecules					
ADAMTS9	Q9P2N4	TS1	ECM remodeling	BM and umbilical cord	24
α Fetoprotein	P02771	DC	Apoptosis prevention	Yolk sac, fetal liver, and placenta	25 and 26
α-2-HS glycoprotein	P02765	EM, WJ-CM, TS1, TS2, and DC	Negative regulation of osteogenesis and TGF-β antagonist	BM, umbilical cord, fetal liver, and all MSC-derived tissue	27 and 28
Collagen α-1 (I) chain	P02452	WJ-CM, TS2, and DC	Ossification, osteoblast differentiation, and regulation of adhesion	BM and umbilical cord	29–31
Collagen α-1 (III) chain	P02461	DC	Osteoblastogenesis and immune regulation	BM and umbilical cord	32–34
Collagen α-2 (I) chain	P08123	WJ-CM, TS2, and DC	Ossification, osteoblast differentiation, and regulation of adhesion	BM and umbilical cord	29 and 30
Collagen α-6 (VI) chain	A6NMZ7	DC	Cell adhesion, regulation of cell differentiation, inhibition of apoptosis, and oxidative damage	BM and umbilical cord	29 and 35
Fibronectin	P02751	WJ-CM, TS2, and DC	Adhesion, migration, growth, differentiation, and bone matrix formation	BM and placenta	34 and 35
FREM3	P0C091	DC	Cell adhesion	BM	36
Glypican 4	O75487	TS1	Cell–cell interaction and control of cell growth and differentiation	BM	37
Hypoxia upregulated protein-1/GRP170	Q9Y4L1	DC	Stress-induced chaperone	BM and placenta	36 and 38
Lactotransferrin	E7EQB2	DC	Promotion of lymphocyte maturation, regulation of myelopoiesis, regulation of bone metabolism, and anti-inflammatory and immunoregulatory proprieties	BM	39–41
MMP8	P22894	TS1	ECM remodeling and hematopoietic stem/progenitor cells mobilization	BM	42
PAI-1	P05121	EM and DC	ECM remodeling and regulation of hematopoietic regeneration	BM	43
Serine carboxypeptidase 1	Q9HB40	DC	ECM remodeling	BM	44 and 45
SPARC	P09486	WJ-CM, TS1, TS2, and DC	ECM remodeling; bone remodeling; osteoblast formation, maturation, and survival; B lymphopoiesis stimulation; development of erythroid progenitors; and angiogenesis	BM (endosteal niche)	46–48
TIMP-1	P01033	WJ-CM, TS2, and DC	ECM remodeling; proliferation, apoptosis, differentiation, and angiogenesis; and regulation of erythroid cells proliferation	BM, umbilical cord, and placenta	49 and 50
Transforming growth factor-β-induced protein ig-h3	Q15582	WJ-CM and DC	Cell adhesion and migration	BM and fetal liver	51–53
Wnt-4	P56705	TS1	Osteogenesis, HSC proliferation, myeloid, and B lymphoid development	BM	54 and 55
Wnt-6	Q9Y6F9	DC	Osteoblastogenesis and macrophage proliferation and differentiation	BM	56
Membrane molecules					
Cadherin-23	Q9H251	DC	Cell–cell interaction	BM	36
CD109/150 kDa TGF-β-1-binding protein	Q6YHK3	DC	Negative regulation of TGF-β responses and osteoclastogenesis	BM ($CD34^+$)	57 and 58
Integrin α4	P13612	DC	Cell adhesion and homing and HSC growth	BM	59
Interleukin 23 receptor	Q5VWK5	WJ-CM	Stimulates T cells, NK cells, and possibly certain macrophage/myeloid cells	BM	60
Lysophosphatidic acid receptor 1	Q92633	DC	Cell migration; regulator of migration, growth, and survival of myeloid progenitors; $CD34^+$ survival; regulation of primitive hematopoiesis; and osteoblast differentiation	BM, yolk sac, and placenta	61 and 62
Notch-2	Q04721	DC	Inhibition of granulocytic differentiation, negative role in megakaryopoiesis, enhance of erythrocytic differentiation, suppressing osteoblastogenesis, and promotion of osteoclastogenesis	BM (hematopoietic progenitors and $CD34^+$) and yolk sac	63–65
Protocadherin 8	O95206	DC	Cell–cell interaction and cell sorting movements	Placenta exosomes	66
Protocadherin β7	Q9Y5E2	DC	Cell–cell interaction	BM	36
Stromal interaction molecule 2	Q9P246	EM and TS1	Macrophage activation, chemotactic cell migration, activation of $CD4^+$, and differentiation of naive $CD8^+$ T cells	BM, $CD4^+$ and $CD8^+$ cells, and placenta	36 and 67
Transporter SLC6A2	H3BM11	DC	Sodium-dependent noradrenaline transporter and bone remodeling	Placenta (RNA)	36 and 68

Note: DC = direct contact; EM = expansion medium; WJ-CM = WJ-MSCs–conditioned medium; TS1 = upper of transwell system; TS2 = bottom of transwell system; BM = bone marrow; WJ-MSCs = Wharton's jelly mesenchymal stem cells.

Colony Forming Capacity of HSPCs Expanded in the DC Condition

We assessed the ability of $CD34^+$ progenitor cells localized in the 2 compartments of the DC system to develop different types of colonies in 6 of the total 12 UCB samples. A higher proportion of primitive colonies, (CFU-GM) and erythroid colonies (BFU-E), was developed from cells of the adherent fraction (P value $= 0.007$) compared to the cells of the respective floating ones (Table 2). Moreover, the most primitive colonies, GEMM colonies (CFU-GEMM) even if represented at very low frequencies in unmanipulated UCB samples (about 1% over the total colonies developed), also exhibited a trend of increase in the adherent cell fraction compared to the floating one (on average 3.5% vs. 0.5%, respectively).

A further confirmation that adherent $CD34^+$ cells are more primitive HSPCs comes from the analysis of BFU-E colony size. The frequency of intermediate/large BFU-E colonies (that consisted of more than 7 clusters) over the total BFU-E colonies was significantly increased in the hematopoietic cells plated from the $CD34^+$ cells adherent to the WJ-MSCs with respect to the floating cell fraction (37.0 ± 14.7 vs. 19.0 ± 6.3, respectively, $P = 0.013$; Table 2).

Proteomic Analysis of Conditioned Media

In order to identify molecular players involved in the cross-talk between $CD34^+$ cells and WJ-MSCs, we analyzed the secretome of different culture media by HPLC-MS/MS. Notably, in our analyses, we did not consider the presence of SCF and FLT-3 ligand, since these cytokines are components of the EM used in all conditions. We identified 166 proteins in the EM, 154 proteins in the upper compartment of the transwell coculture system (TS1), 114 proteins in the lower compartment of the transwell coculture system (TS2), 133 proteins in the DC, and 105 proteins in the WJ-CM. The TS medium was analyzed both in the upper and lower compartments in order to discriminate molecules that may be distinctively produced by hematopoietic and/or stromal cells, therefore not passing through the pores of the transwell PET membrane for different reasons such as extracellular deposition.

Since our experiments showed that the $CD34^+$ cells can establish different interactions with WJ-MSCs (i.e., the presence of an adherent and a floating cell fraction within DC), we focused our analysis on extracellular proteins that can recreate in vitro a microenvironment similar to that of a hematopoietic niche. We highlighted the presence of 30 extracellular/exosomal or membrane proteins, listed in Table 3, and we performed a comparative analysis among tested culture conditions to investigate the expression differences[24–68]. Interestingly, in the DC secretome, we identified a set of 16 exclusively expressed proteins. Only some of them were already described in any of the known hematopoietic niches, either prenatal or adult, with most data coming from studies on BM.

Structural molecules with cytoadhesive roles such as collagens, namely, types I, III, and VI, were detected in the secretome of the WJ-MSCs in the 2 coculture conditions (DC and TS2). Confirming the data of ICC analyses shown before, here for the first time, we report the presence of fibronectin as player in cross talk with HSPCs. In fact, secretomic data have allowed detection of fibronectin expression in WJ-CM, DC, and TS2.

Interestingly, specifically in the DC system, we found for the first time the expression of Frem3 a molecule involved in cell adhesion reported to be expressed in BM[36]. We also detected in the DC, WJ-CM, TS2 systems, tissue inhibitor of metalloproteinases-1 (TIMP-1) a protein directly involved in the finely regulated remodeling of the BM extracellular matrix[49] (Table 3).

We detected for the first time specifically in the DC condition the α-fetoprotein, well known to be expressed in in vivo prenatal hematopoietic niches.

Here, we report for the first time the presence of lactotransferrin (iron binding and multifunction protein) in the DC system.

Serine carboxypeptidase-1 (SCPEP1) is a secreted serine protease which has been implicated in monopoiesis[44]. Interestingly, in our system, this molecule was expressed only in the DC secretome. We are the first to show SCPEP1 expression by human WJ-MSCs because only one literature report (to the best of our knowledge) demonstrated its expression in porcine WJ-MSCs[45].

Wnt-4 and Wnt-6 are part of the recently discovered noncanonical Wnt-Ca^{2+} pathway which is active in hematopoiesis[55]. No data are present in literature on the expression of these molecules in coculture systems with WJ-MSCs. Here, we demonstrated that Wnt-4 was specifically found in TS1, while Wnt-6 was present in the DC secretome. Noteworthy in the DC system, we identified the presence of Notch2. Wnt and Notch signaling are integrated and are selectively active in regulating hemopoiesis and have been implicated in erithropoiesis[63] and in supporting ex vivo expansion of HSPCs. Transforming growth factor-beta (TGF-β) pathways are involved in the regulation of the HSPC homeostasis. Interestingly, we found the TGF-β induced protein H3 (BIGH3) in the DC and WJ-CM secretomes.

Moreover, we detected the secreted molecule α-2 HS glycoprotein (Fetuin A) in all the tested coculture conditions. Importantly, this molecule binds several growth factors (such as TGF-β) regulating their access to surface receptors[27]. Finally, CD109 is a glycosylphosphatidylinositol-anchored glycoprotein that binds the TGF-β receptor. Here, we showed that CD109 was detected only in the DC culture system (Table 3).

Discussion

In the present article, we investigated how WJ-MSCs or their soluble factors influenced the kinetics of UCB-HSPC expansion and which type of hematopoietic subpopulations was

amplified in vitro. Our experiments were performed in a relatively short time to avoid extensive manipulation of HSPCs in order to set ex vivo expansion protocols suitable for future clinical applications.

Our data showed that the DC system sustained a greater $CD34^+$ cell expansion rate with respect to the other tested conditions. Moreover, we here report for the first time that

i. $CD34^+$ cells interacted with WJ-MSCs in different fashions allowing the detection of a floating and an adherent fraction;
ii. WJ-MSCs polymerized an intricate fibronectin-rich network, directing the arrangement of the $CD34^+$ cells and providing a microenvironment which favored the interaction between the 2 cell types;
iii. the DC system recapitulates some functional aspects of a hematopoietic niche. In fact, it is able to expand a component of primitive cells, with phenotypic features typical of long-term repopulating cells, and also to greatly increase the number of lineage-committed shorter-term repopulating cells.

These results are relevant for the outcome of hematopoietic stem cell transplantation.

In fact, the more primitive cells have been linked to the long-term engraftment of a transplant, while an expansion product with more mature committed progenitors and mature cells will be needed to facilitate engraftment to a clinically relevant degree[69].

In addition, adherent $CD34^+$ cells were able to develop a higher frequency of primitive colonies, CFU-GM and large BFU-E, and also of the most primitive CFU-GEMM in comparison to the floating cells.

Relevant to these results, we observed a rare hematopoietic stem cell (HSC) population (identified by other authors[70] for the coexpression of CD34/CD90 and CD49f and the lack of CD38 and CD45RA) that was more represented in the adherent cell fraction (unpublished observation).

It is important to note that we showed that the WJ-CM also promoted the ex vivo expansion of $CD34^+$ cells, even if to a lesser extent than the cell–cell contact system. In particular, WJ-CM sustained the growth of multipotent hematopoietic progenitors. This finding can be relevant in clinical translation. Further experiments will be needed to better scale up and define this system.

Overall, these functional data revealed that both WJ-MSCs and their soluble factors may be important tools to increase the number of UCB-$CD34^+$ cells in ex vivo systems.

WJ-MSCs physiologically contribute to create the microenvironments that sustain hematopoiesis during the embryonic and fetal period of life. A better characterization of factors produced by WJ-MSCs in the different culture systems may therefore provide important insights on the future expansion strategies employed for UCB-derived $CD34^+$ cells.

The secretomic analysis of the different tested culture conditions revealed the presence of specific molecules that may be potentially be involved in the observed supporting effects on $CD34^+$ cell expansion. The polymerization of an active extracellular matrix already suggested by fibronectin immunolocalization is further validated by the identification of several other adhesion molecules, such as collagens type I, III, and VI, which were consistently found in the DC condition. These molecules together with fibronectin and SPARC are known to be part of the BM and UC and placental hematopoietic niches[29,31–33,46,47].

We also found proteins expressed in prenatal hematopoietic sites such as yolk sac and fetal liver and involved in hematopoiesis, namely alpha-fetoprotein (AFP) and lactotrasferrin. It is also important to note that it has been reported that MSC-derived AFP prevents apoptosis of HSPCs by cooperating with SCF[26].

Wnt signaling pathway molecules were also found in our cocolture system. In hematopoietic cells, Wnt proteins may function as proliferation-inducing growth factors, and also affect cell-fate decisions, similar to true morphogens in other systems[54,55].

Interestingly, a number of TGF-β interacting molecules (BIGH3, Fetuin A, and CD109) were found in the secretomes of our coculture system. Multiple reports have shown that TGF-β members regulate tissue homeostasis and regeneration. With respect to hematopoiesis, TGF-β plays an important role in regulating HSPC behavior, particularly regarding quiescence and self-renewal[71]. BIGH3 has been reported to be part of the hematopoietic (BM) niche by mediating the adhesion of HSPCs, which can bind this molecule both via integrin-dependent and integrin-independent adhesion[51]. As a modulator of HSPC adhesion and migration, competing for fibronectin binding sites in adhesion assays, BIGH3 may be essential to loosen the contacts of HSCs with their originating niche, rendering them susceptible to polarization and subsequent egress[52].

Fetuin A is known to be expressed in embryonic/fetal tissues (such as fetal liver). In addition to its known role in binding TGF-β receptor, CD109 was reported as part of the exosomal fraction. Moreover, a secreted furin-cleaved CD109 fragment can modulate TGF-β signaling in adjacent cells[58]. It is particularly intriguing that negative regulators of TGF-β activity, such as fetuin A and CD109, are expressed in this in vitro expansion system and specifically in the DC culture. Therefore, present data suggest that regulation of the TGF-β pathways may in part explain the results obtained so far in our DC system.

Conclusion

Our results showed that both WJ-MSCs and their conditioned medium are capable of sustaining in vitro hematopoiesis and can be used as a tool to promote HSPC expansion for clinical applications. We also provided insights on the possible mechanisms behind the hematopoiesis support

function of WJ-MSCs. Interestingly, the secretomic analysis showed that the molecules expressed in the DC system recapitulated a functional niche-like hematopoietic microenvironment, a finding further supported by morphological analyses reported here. We highlighted that the DC system specifically provides the expansion of either multipotent or committed progenitor cells, both essential for the outcome of HSPC transplantation. To this end, clinical trials already demonstrated that co-transplantation of UCB-HSPCs and WJ-MSCs generates a superior graft with respect to UCB alone, as reviewed in Reference 9. Further studies are needed to better delineate the roles of the molecular players that we identified in our experiments.

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Ethical Approval

This study was approved by our institutional review board. Approval case numbers no. 124 (01-12-2010) and no.331 (8-11.2016).

Statement of Human and Animal Rights

Human rights were treated in accordance with the tenets of the Declaration of Helsinki and local ethical regulations.

Statement of Informed Consent

Human samples were obtained after acquiring written informed consent from the mothers.

Declaration of Conflicting Interests

The author(s) declared the following potential conflicts of interest with respect to the research, authorship, and/or publication of this article: Giampiero La Rocca is a member of the Scientific Board of Auxocell Laboratories, Inc. The other authors declare no potential conflicts of interest.

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