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Human Umbilical Cord Wharton's Jelly Stem Cells and Its Conditioned Medium Support Hematopoietic Stem Cell Expansion Ex Vivo

C.Y. Fong, K. Gauthaman, S. Cheyyatraivendran, H.D. Lin, A. Biswas, and A. Bongso*

Department of Obstetrics and Gynaecology, Yong Loo Lin School of Medicine, National University of Singapore, Kent Ridge, Singapore 1190741, Singapore

ABSTRACT

Bone marrow mesenchymal stromal cells (BMMSCs) have been used as feeder support for the ex vivo expansion of hematopoietic stem cells (HSCs) but have the limitations of painful harvest, morbidity, and risk of infection to the patient. This prompted us to explore the use of human umbilical cord Wharton's jelly MSCs (hWJSCs) and its conditioned medium (hWJSC-CM) for ex vivo expansion of HSCs in allogeneic and autologous settings because hWJSCs can be harvested in abundance painlessly, are proliferative, hypoimmunogenic, and secrete a variety of unique proteins. In the presence of hWJSCs and hWJSC-CM, HSCs put out pseudopodia-like outgrowths and became highly motile. Time lapse imaging showed that the outgrowths helped them to migrate towards and attach to the upper surfaces of hWJSCs and undergo proliferation. After 9 days of culture in the presence of hWJSCs and hWJSC-CM, MTT, and Trypan blue assays showed significant increases in HSC numbers, and FACS analysis generated significantly greater numbers of $CD34^+$ cells compared to controls. hWJSC-CM produced the highest number of colonies (CFU assay) and all six classifications of colony morphology typical of hematopoiesis were observed. Proteomic analysis of hWJSC-CM showed significantly greater levels of interleukins (IL-1a, IL-6, IL-7, and IL-8), SCF, HGF, and ICAM-1 compared to controls suggesting that they may be involved in the HSC multiplication. We propose that cord blood banks freeze autologous hWJSCs and umbilical cord blood (UCB) from the same umbilical cord at the same time for the patient for future ex vivo HSC expansion and cell-based therapies. J. Cell. Biochem. 113: 658–668, 2012.

KEY WORDS: HEMATOPOIETIC STEM CELL EXPANSION; CONDITIONED MEDIUM; WHARTON'S JELLY STEM CELLS

Bone marrow hematopoietic stem cell (HSC) transplantation has been used for the treatment of malignant hematopoietic diseases. However, the aspiration of HSCs from the bone marrow is painful with the potential risk of infection and morbidity, and optimal HSC numbers are not always available for successful transplantation in many cases. To avoid these disadvantages, HSCs from the human umbilical cord blood (UCB) have been successfully used for the treatment of both malignant and non-malignant hematopoietic diseases in children in autologous and allogeneic settings [Rubinstein et al., 1998; Laughlin et al., 2001]. UCB contains HSCs and hematopoietic progenitor cells (HPCs) that appear to have higher proliferation rates and immunological tolerance compared to those in bone marrow [Gluckman et al., 1989]. Unfortunately, UCB also has its limitations in that the HSC and HPC yields can be low and the cell numbers adequate for the treatment of hematopoietic diseases in children and not adults [Paulin, 1992]. It is estimated that for successful engraftment, at least 2.5×10^6 $CD34^+$ cells per kg of patient body weight is required [Rocha et al., 2000] but a good UCB harvest from a single umbilical cord generates only about 10×10^6 $CD34^+$ cells which is adequate for only a 4 kg child [Zhang et al., 2006].

Several approaches have been suggested to alleviate the problems of inadequacy of HSC numbers for transplantation. These include (a) administration of a second UCB unit to the patient from another donor or (b) ex vivo expansion of the same patient's UCB-HSCs. The latter approach is more favorable for immunological reasons as cell rejection can be avoided when autologous HSCs are administered. However, any expansion protocol must attempt to simulate as close as possible in vivo hematopoiesis while maintaining the stemness properties of the HSCs.

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*Correspondence to: Prof. A. Bongso, Department of Obstetrics and Gynaecology, Yong Loo Lin School of Medicine, National University of Singapore, Kent Ridge, Singapore 1190741, Singapore. E-mail: obgbongs@nus.edu.sg

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Human mesenchymal stem cells (MSCs) have been successfully used in vitro as a scaffold for stromal support and expansion of HSCs via cell-to-cell contact. This concept was developed on the understanding that MSCs exist within the bone marrow in vivo and act as a natural scaffold on which the in vivo HSCs interact and proliferate. It is not definitely known whether the mechanism behind the HSC–MSC interaction that results in HSC proliferation is mediated by diffusible factors crossing over from the MSCs to HSCs during cell-to-cell contact or through secretions by the MSCs into the immediate microenvironment of the HSCs. To avoid immunological complications, autologous MSCs from the same patient's bone marrow or UCB have been used as a scaffold in vitro, for expansion of her own HSCs with successful results [Zhang et al., 2006; Alakel et al., 2009]. The use of MSCs from UCB for stromal support of autologous HSCs has its own limitations in that the numbers of MSCs in UCB are extremely low and their existence in UCB has been controversial [Mareschi et al., 2001; Wexler et al., 2003]. Musina et al. [2007] reported very low counts of UCB MSCs per volume of UCB and showed that such MSCs had low proliferation rates.

Large numbers of bona fide, fully characterized MSCs with high proliferation rates and low population doubling times have been reported in the human umbilical cord Wharton's jelly (hWJSCs) by several workers including our group [Wang et al., 2004; Weiss et al., 2006; Fong et al., 2007, 2010; Chao et al., 2008]. We showed that at least 4.6×10^6 fresh live hWJSCs can be harvested from 1 cm of umbilical cord [Fong et al., 2010] and the stemness properties of these hWJSCs lasted longer than bone marrow MSCs in vitro (10 passage vs. 3 passage) [Fong et al., 2010]. hWJSCs were also shown to be hypoimmunogenic, thus being able to be used in both autologous and allogeneic settings without the concerns of graft versus host disease [Weiss et al., 2008] and thaw-survival rates of hWJSCs after cryopreservation were >90% [Fong et al., 2010]. Given these unique beneficial properties and the ready supply of hWJSCs that can be harvested painlessly, we suggest that hWJSCs could serve as an attractive source of MSCs for stromal coculture support for the expansion of HSCs when compared to other MSC sources. Furthermore, since the conditioned medium of cultured hWJSCs (hWJSC-CM) contains a variety of beneficial growth factors secreted by the hWJSCs such as interleukins and growth factors [Friedman et al., 2007; Bakhshi et al., 2008], it is possible that hWJSC-CM may provide good support for HSC expansion and as such its use may require less stringent approval from regulatory bodies for clinical application.

We therefore undertook studies to evaluate the use of allogeneic and autologous hWJSCs and hWJSC-CM for ex vivo expansion of UCB HSCs.

MATERIALS AND METHODS

DERIVATION, PROPAGATION, AND CHARACTERIZATION OF hWJSCs

Several groups have derived MSCs from various compartments of the human umbilical cord such as the amniotic membrane [Illancheran et al., 2007], subamnion [Kita et al., 2010], perivascular areas surrounding the umbilical blood vessels [Sarugaser et al., 2005], and Wharton's jelly [Weiss et al., 2006; Fong et al., 2007] and have referred to such MSCs vaguely as umbilical cord matrix stem cells (UCMSCs). Whether the nature and properties of the MSCs from all these sources are the same is not known. Furthermore, the methods used for derivation of MSCs from the human umbilical cord have also been drastically different between groups with some groups removing the umbilical blood vessels and then scraping off the Wharton's jelly from the inner lining of the umbilical cord [Wang et al., 2004], while others keep the umbilical blood vessels intact before scraping off the Wharton's jelly [Romanov et al., 2003]. Some groups tie the ends of the removed umbilical blood vessels into loops and immerse them into an enzymatic solution to collect MSCs from the perivascular areas [Sarugaser et al., 2005] while some others remove the blood vessels and then dice up the umbilical cord pieces into smaller pieces and immerse them into enzymatic solutions to retrieve MSCs [Karahuseyinglu et al., 2007]. Based on the method used there is therefore the possibility of heterogeneous populations of cells being harvested. To therefore obtain a more defined homogeneous population of MSCs directly from the Wharton's jelly with minimum or no contamination from other compartments we developed our own method of derivation [Fong et al., 2007, 2010].

Approximately 15–30 cm long pieces of human umbilical cords were collected after informed patient consent and approval from the Singapore Ministry of Health Institutional Domain Specific Review Board (DSRB). Briefly, the human umbilical cord pieces were cut into smaller 3 cm pieces and washed in Hank's balanced salt solution (HBSS, Invitrogen Life Technologies, Carslbad, CA). Each small piece was slit open with sterile forceps and curved scissors and their inner surfaces containing the attached Wharton's jelly were inverted face down into a Petri dish containing a small volume (1.5 ml) of an enzymatic solution that allowed only the Wharton's jelly to come into contact with the enzymes. The enzymatic solution comprised of collagenase type I, collagenase type IV, and 100 IU of hyaluronidase (Sigma Chemical Co, USA) in DMEM medium (Invitrogen Life Technologies). The Petri dishes were then incubated at 37°C in a 5% CO_2 in air atmosphere for 45 min to allow loosening and separation of the Wharton's jelly. Any remaining Wharton's jelly that was still attached to the inner surfaces of the cord pieces was carefully separated into fresh medium using the blunt surface of a pair of curved forceps and mixed with the Wharton's jelly in the previous Petri dishes. The final solution containing the gelatinous Wharton's jelly was transferred to a new Petri dish containing 3 ml of fresh DMEM medium and the Wharton's jelly syringed through an 18 G needle to further break up the gelatinous masses to release the hWJSCs. The solution was then collected into sterile 15 ml tubes, centrifuged at 300*g* for 10 min, supernatant discarded, and cell pellets resuspended in a hWJSC medium containing 80% DMEM high glucose supplemented with 20% knockout (KO) serum replacement (Invitrogen Life Technologies), 16 ng/ml basic fibroblast growth factor (Millipore Bioscience Research Agents, Temecula, CA), 1% non-essential amino acids, 2 mM L-glutamine, 0.1 mM β-mercaptoethanol, 1% insulin-transferrin-selenium (ITS), antibiotic-antimycotic mixture (Invitrogen Life Technologies). The cells were then seeded into sterile T25 plastic tissue culture flasks [Becton Dickinson (BD), USA] and incubated at 37°C in a 5% CO_2 in air atmosphere. When the cells reached confluence in primary

culture in approximately 6–7 days, they were passaged by detachment and disassociation with trypsin–EDTA (Invitrogen Life Technologies), centrifugation at 300*g* for 10 min, seeding of cell pellets into new T25 flasks and incubation. The phenotype and growth of primary and passaged cells were monitored daily and photographed under inverted phase contrast optics.

The hWJSCs were characterized used the full battery of conventional characterization tests prescribed for MSCs [Dominici et al., 2006; Fong et al., 2010]. For all experiments in this study early passaged hWJSCs (P3) were used.

PROPAGATION OF HSCs

Approval for purchase and use of commercial cord blood $CD34^+$ HSCs was given by the National University of Singapore Institutional Review Board (NUS-IRB). The frozen $CD34^+$ HSCs were thawed using the instructions and consumables supplied by the manufacturer (Stem Cell Technologies, Inc., Singapore). Briefly, the frozen cells were first thawed in a water bath (37°C) and quickly transferred to a conical tube containing DNase 1 to prevent cell clumping. Approximately, 15 ml of the provided medium [Iscove's MDM (IMDM) containing 10% fetal bovine serum (FBS)] was added into the tube, the solution gently resuspended and then centrifuged at 200*g* for 15 min at room temperature. The supernatant was decanted, and cell pellet seeded separately into two types of culture media, basal medium (BM), and enriched medium (EM). BM (Stemspan, Serum-free expansion medium, SFEM) comprised of Iscove's MDM medium (IMDM) supplemented with bovine serum albumin (BSA), recombinant human insulin, human transferrin, 2-mercaptoethanol, and other supplements. Recombinant hematopoietic growth factors have not been added to this commercial BM. The individual concentrations of these ingredients and the nature of the supplements were not disclosed by the manufacturer (Stem Cell Technologies, Inc.). The composition of EM was the same as BM but additionally supplemented with CC110 cytokine cocktail which contained a combination of recombinant human cytokines to support the proliferation of human HPCs. The nature and concentrations of the individual cytokines were not disclosed by the manufacturer (Stem Cell Technologies, Inc.). The FACS profile given from the company showed that more than 90% of the cells were $CD34^+$.

The autologous $CD34^+$ HSCs were isolated from up to 100 ml of UCB which was aspirated from the umbilical blood vessels of the same umbilical cords providing hWJSCs using 50 ml syringes and large bore needles. Approval for use of these autologous HSCs was given by the Singapore Ministry of Health Institutional Domain Specific Review Board (MOH-DSRB). The UCB HSCs were isolated using Ficoll–Paque (Stem Cell Technologies, Inc.) density gradient centrifugation according to a method described by Jaatinen and Laine [2007]. Briefly, the UCB was diluted in PBS/EDTA diluents in the ratio of 1:4 and carefully over-layered on the Ficoll–Paque solution and then centrifuged for 40 min. The buffy coat interface layer was collected and washed. $CD34^+$ cells were then enriched from the buffy coat by immunomagnetic positive selection using the MidiMACS^{TM} system (Miltenyi Biotec, Auburn, CA) according to the manufacturer's instructions. Briefly, the mononuclear cells were labeled with CD34 microbeads and added into a MACS LD column attached to a MidiMACS^{TM} magnetic separator. The LD column was then removed from the MidiMACS^{TM} separator and the bound $CD34^+$ cells were eluted with a plunger.

COCULTURE OF COMMERCIAL HSCs WITH ALLOGENEIC hWJSCs

Donor allogeneic hWJSC monolayers (80% confluent) were first inactivated by exposure to 20 μg/ml mitomycin-C (MMC, NUH Pharmacy, Singapore) and incubated at 37°C for 2.5 h. After incubation, the hWJSCs were washed thoroughly twice with phosphate-buffered saline (PBS) followed by one wash with hWJSC medium. The cells were then disassociated with trypsin–EDTA (Invitrogen Life Technologies) and 25,000 inactivated hWJSCs were seeded per well in two sets (A and B) of 24-well plates (Nalge Nunc International, Rochester, NY). After 24 h when the hWJSCs had attached to the plastic surfaces of the wells in both sets of plates (60–70% confluence), the hWJSC medium was completely removed and an equal number of commercial $CD34^+$ HSCs (25,000 in 0.5 ml of BM) were seeded in each well of Plates A and similar numbers of HSCs in 0.5 ml of EM seeded in each well of Plates B. Plates A (hWJSC-BM) and B (hWJSC-EM) were incubated for 9 days at 37°C in a 5% CO_2 in air atmosphere with topping up with 0.25 ml of BM or EM every 48 h, after which the HSCs were subjected to a battery of assays to evaluate cell behavior and proliferation.

CULTURE OF COMMERCIAL HSCs WITH ALLOGENEIC hWJSC CONDITIONED MEDIUM (hWJSC-CM)

Early passaged hWJSCs (3P) were first grown to 70% confluence in hWJSC medium followed by removal of the spent medium. The monolayers were then washed with PBS twice to remove any residual hWJSC medium and the cells grown in BM for 24 h after which the conditioned BM was separated. This medium was referred to as basal conditioned medium (BCM). The same procedure was carried out with EM and this conditioned medium was referred to as enriched conditioned medium (ECM). Both BCM and ECM were filtered by passing through a 0.22 μm filter. Freshly thawed commercial $CD34^+$ HSCs (25,000) were seeded in each well of 24-well plates (Nalge Nunc International) and grown separately in 0.5 ml of BCM and ECM for 9 days at 37°C in 5% CO_2 in air with topping up with 0.25 ml of BCM or ECM every 48 h.

After 9 days of culture, the $CD34^+$ HSCs were subjected to the same battery of assays as the coculture plates above to evaluate cell behavior and proliferation. Commercial $CD34^+$ HSCs and MMC-treated hWJSCs were cultured alone and in parallel with the experimental plates for 9 days in the four different culture media (BM, BCM, EM, and ECM) to act as controls.

CULTURE OF AUTOLOGOUS HSCs WITH hWJSCs AND hWJSC-CM FROM THE SAME UMBILICAL CORDS

Twenty-five thousand MMC-treated hWJSCs were seeded in each well of 24-well culture plates (Nalge Nunc International) containing EM and incubated at 37°C in a 5% CO_2 in air atmosphere. When the hWJSCs had adhered in each well (60–70% confluence), the medium was removed and an equal number of autologous HSCs (in 0.5 ml of EM) that were harvested from the same umbilical cord as the hWJSCs, were added to each well and incubated for 9 days with topping up with 0.25 ml of fresh EM every 48 h. Separately,

25,000 autologous HSCs in 0.5 ml of ECM were seeded into each well of 24-well plates (Nalge Nunc International) and incubated at 37°C in a 5% CO_2 in air atmosphere and incubated for 9 days with topping up with 0.25 ml of fresh ECM every 48 h. After 9 days, the autologous HSCs in both hWJSC and hWJSC-CM plates were subjected to the MTT assay for evaluation of cell proliferation and FACS analysis for calculation of $CD34^+$ cell counts.

PHASE CONTRAST OPTICS AND SCANNING ELECTRON MICROSCOPY (SEM)

The interaction of the commercial and autologous HSCs with the hWJSCs and hWJSC-CM were studied using time lapse imaging, phase contrast optics and conventional scanning electron microscopy (SEM). Videos and images of the behavior of the cells were captured using a digital camera at regular intervals when the cells were monitored during the 9 days of culture.

CELL PROLIFERATION: MTT ASSAY

Ten microliters MTT reagent (final concentration of 0.5 mg/ml) was added to the culture media in the hWJSC and hWJSC-CM plates (A and B) after the 9 days of exposure to HSCs, and the plates were incubated for 4 h until the appearance of visible purple precipitate. The plates were then centrifuged at 300*g* for 5 min to spin down the HSCs, medium decanted, and 100 μl of detergent reagent added into each well. The plates were incubated in the dark at 37°C in a 5% CO_2 in air atmosphere for 2 h and absorbance measured at 570 nm against a reference wavelength of 630 nm using a spectrophotometer equipped with micro plate ELISA reader.

TRYPAN BLUE VITAL CELL COUNTS

Aliquots of the cultured $CD34^+$ HSCs from the hWJSC, hWJSC-CM and control plates after 9 days of culture were taken and stained with 0.4% Trypan Blue (vital dye) (Sigma Chemical Co) for 1 min at room temperature. The number of live HSCs (unstained) were counted using a hemocytometer.

FLOW CYTOMETRIC ANALYSIS

The cultured $CD34^+$ cells from the hWJSC, hWJSC-CM, and control plates were blocked with 10% normal goat serum (NGS) (Invitrogen Life Technologies) to prevent non-specific binding and then incubated with primary antibodies (1:100) for the CD34 marker (Biolegend, San Diego, CA) for 30 min, followed by a PBS wash and incubation with Alexa Fluor®488 (1:750) secondary antibody for 30 min (Invitrogen Life Technologies). The cells were then re-suspended in 10% NGS, filtered through a 40 μm nylon strainer to remove any cell clumps and analyzed using a CyAn™ ADP Analyzer (Beckman Coulter, Fullerton, CA).

COLONY FORMING ASSAY

The cultured $CD34^+$ cells from the hWJSC, hWJSC-CM, and control plates were separated, centrifuged 300*g* for 5 min, supernatant decanted, and cell pellets seeded into wells of 24-well plates (Nalge Nunc International) containing 0.5 ml of semisolid methylcellulose in Methocult H4435 medium. This medium was a commercial medium from Stem Cell Technologies, Inc. and contained IMDM, BSA, 2-mercaptoethanol, recombinant human stem cell factor (rhSCF), granulocyte colony stimulating factor (rhG-CSF), granulocyte macrophage-CSF (rhGM-CSF), interleukin-3 (rhIL3), interleukin-6 (rhIL6), erythropoietin (rhEPO), and supplements. The individual concentrations of each supplement was not disclosed by the manufacturer. Six different cell densities ranging from 300 to 10,000 cells were seeded per well and plates incubated at 37°C in a 5% CO_2 in air atmosphere. After 14 days, colonies that were formed were counted and analyzed for morphology. Colony morphologies were classified according to that described in the manufacturer's manual (Stem Cell Technologies, Inc. Instruction Manual) as (1) colony forming unit-erythroid (CFU-E), (2) colony forming unit-granulocyte (CFU-G), (3) colony forming unit-granulocyte/macrophage (CFU-GM), (4) burst forming unit-erythroid (BFU-E), (5) colony forming unit-macrophage (CFU-M), and (6) colony forming unit-granulocyte/erythrocyte/macrophage/megakaryocyte (CFU-GEMM). Colony forming unit numbers were calculated by dividing the number of colonies at day 14 by the number of cells plated and multiplying this value by 10,000 which reflected the colony forming ability of 10,000 cells.

CYTOKINE ANALYSIS OF hWJSC-CM USING THE MULTIPLEX LUMINEX® BEADS ASSAY

Differential cytokine analysis of BCM and controls was carried out using the Bio-Rad Express assay kit for human group I and II cytokines (Bio-Rad Laboratories, Singapore Pte Ltd). The 96-well microtiter plates provided with the kit were wetted with 100 μl of wash buffer and 50 μl of beads were added to each well. The BCM samples (50 μl) were diluted in equal volumes of assay diluent and 50 μl of the diluted sample and standards was added to the beads in each well. The plates were incubated for 1 h at room temperature on a shaker in the dark and the assays were run in duplicate. After incubation the plates were washed twice in buffer and 100 μl of secondary biotinylated antibody (1:10 dilution in antibody diluent) provided with the kit was added to each well. The plates were further incubated at room temperature for 1 h in the dark and then washed twice with buffer. Then 100 μl of streptavidin-PE provided with the kit was added to each well and the plates incubated for 30 min at room temperature in the dark. The wells were finally washed thrice, filled with 100 μl of wash buffer, and the plates then incubated for 2–3 min at room temperature in the dark. The plates were then read on a Bio-plex array reader and data subsequently analyzed using the Bio-plex manager software, version 3.

STATISTICS

All results were expressed as mean ± SEM and statistically significant differences between different groups were calculated using the two-tailed Student's *t*-test (SPSS 13). A value of $P < 0.05$ was considered as statistically significant.

RESULTS

CULTURE OF COMMERCIAL HSCs WITH ALLOGENEIC hWJSCs/hWJSC-CM

Cell behavior. Human Wharton's jelly stem cells derived by the method in this study displayed a short-fibroblast-like morphology

with short population doubling times of approximately 24 h and high proliferation. They met the criteria for MSCs as recommended by the International Society of Cellular Therapy [Dominici et al., 2006]. They were plastic adherent, could be differentiated into several lineages and had high expression levels of CD73, CD13, CD29, CD44, CD90, CD105, and D146, and low level expression for CD45, CD10, CD14, CD34, CD117, and HLA-DR surface molecules. They retained their stemness characteristics for up to 10 passages when characterized with the full battery of characterization tests for MSCs. Mitomycin-C was effective in stopping their multiplication and keeping them as 70–80% confluent monolayers for the purpose of this study.

Over 90% of the commercial HSCs that were purchased tested positive for CD34 markers at the time of this study. Time lapse imaging over a 12 h period and phase contrast optics showed that most of the HSCs changed from a circular to elongated morphology, put out pseudopodia-like outgrowths and migrated towards the bodies of the hWJSCs, loosely attaching to them and undergoing mitosis (Fig. 1A,B). They also became active and showed similar elongated morphology with pseudopodia-like outgrowths no sooner they were exposed to hWJSC-CM (Fig. 1C). Such HSC behavior lasted throughout the 9-day period and was not seen in controls (Fig. 1D). Scanning electron micrographs confirmed the mitotic activity of the HSCs on the hWJSCs and showed that most of the

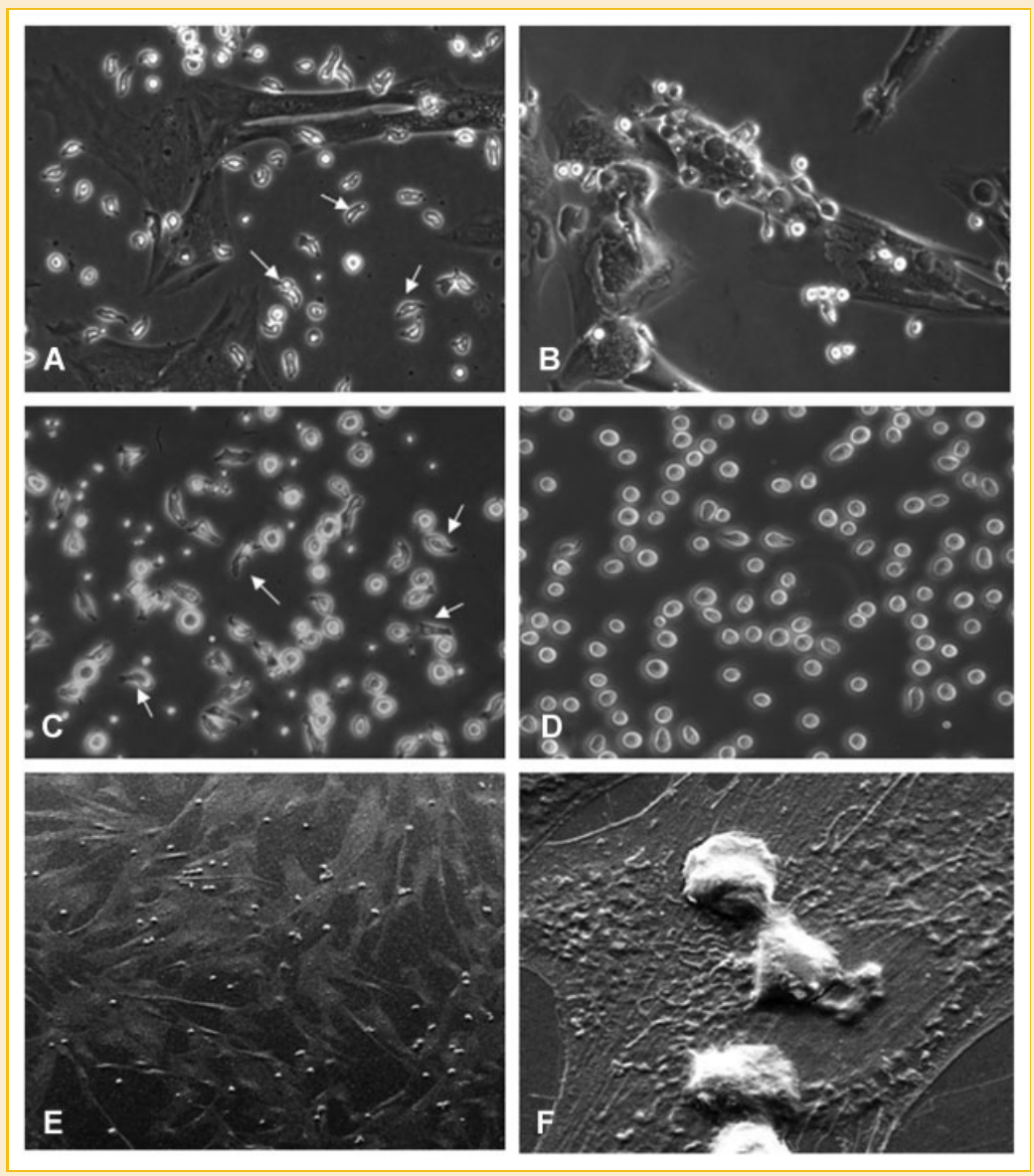

Fig. 1. A: Phase contrast image showing HSCs putting out pseudopodia-like outgrowths (arrows) in the presence of adherent hWJSCs (200×). B: Phase contrast image showing HSCs migrating towards the upper surfaces of hWJSCs, attaching to them and undergoing multiplication (200×). C: Phase contrast image showing several HSCs putting out pseudopodia-like outgrowths (arrows) in hWJSC-CM (200×). D: Phase contrast image showing very few HSCs having pseudopodia-like outgrowths in controls (BM/EM) (200×). E: Scanning electron micrograph (low magnification) showing HSCs loosely attached to upper surfaces of hWJSC monolayers. F: Scanning electron micrograph (high magnification) showing HSCs undergoing mitosis on surface of hWJSC.

HSCs attached to the upper surface of the hWJSC monolayer with very few cells migrating beneath (Fig. 1E,F).

MTT Assay. The mean ± SEM HSC proliferation rates in the presence of hWJSC-BM and BCM were significantly greater than controls (Fig. 2A). The proliferation rates for hWJSC-EM and ECM were also greater than their controls and was significant (Fig. 2A).

Live cell counts (Trypan blue staining). When Trypan blue staining was used to evaluate the vitality of the expanded HSCs, the overall mean ± SEM live HSC counts (including $CD34^+$ cell counts) in the hWJSC-BM and BCM culture environments were significantly greater than controls (Fig. 2B). The overall mean ± SEM live HSC counts for hWJSC-EM and ECM were not significantly greater than their controls but the $CD34^+$ cell counts were significantly different (Fig. 2C).

Flow cytometric analysis (FACS). FACS analysis showed that the hWJSCs and hWJSC-CM culture environments yielded greater numbers of $CD34^+$ HSCs compared to controls (hWJSC-BM: 93.91%; BCM: 96.91%; hWJSC-EM: 96.07%; ECM: 94.71%; BM: 86.68%; EM: 52.97%) (Fig. 2D).

Colony forming unit (CFU) assay. The CFU assay showed that the mean ± SEM number of colonies in the hWJSC-BM (688 ± 125) and BCM (864 ± 272) culture environments were greater than their controls (304 ± 34) (Fig. 3A,C). The hWJSC-EM (656 ± 102) culture environment also generated a greater number of $CD34^+$ colonies than its controls (528 ± 11) but these values were not as high as those with BCM (Fig. 3B). All six types of colony morphology for normal hematopoiesis (CFU-E, CFU-G, CFU-GM, BFU-E, CFU-M, and CFU-GEMM) were observed in the experimental arms compared to controls (Fig. 3D).

CULTURE OF AUTOLOGOUS HSCs WITH hWJSCs/hWJSC-CM FROM THE SAME UMBILICAL CORDS

When fresh unfrozen autologous HSCs from the same patient's umbilical cord were cultured in the presence of its own inactivated hWJSCs or its hWJSC-CM for 9 days using the same protocols as for the commercial HSCs, similar trends in cell behavior, cell proliferation, colony counts, and colony morphology for $CD34^+$ cells were observed (Fig. 4A,B).

PROTEOMIC PROFILES IN hWJSC-CM

Concentration levels of certain members of the interleukin family (IL-1a, IL-6, IL-7, and IL-8), hepatocyte growth factor (HGF), stem cell factor (SCF), monocyte chemotactic protein-1 (MCP-1), and inter-cellular adhesion molecule 1 (ICAM-1) were significantly increased in the BCM compared to controls (Fig. 5). Of these factors, the levels for IL-6, IL-8, HGF, SCF, and ICAM-1 were extremely

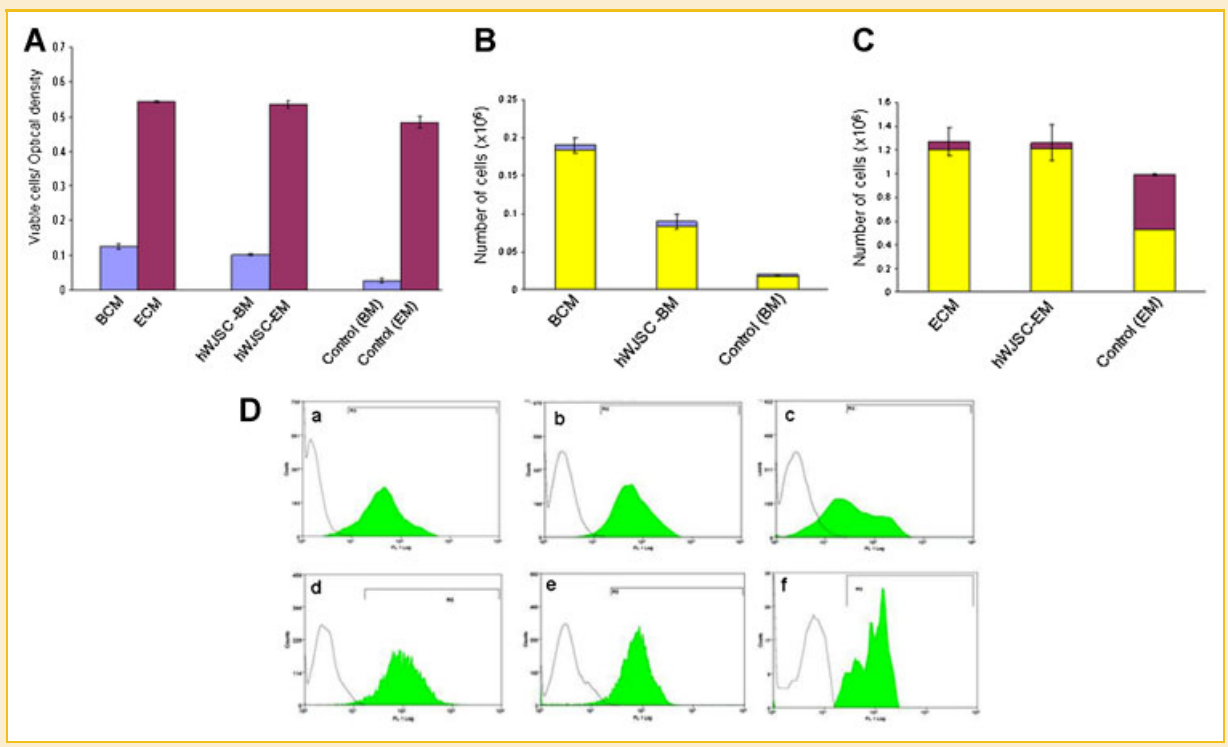

Fig. 2. A: Mean ± SEM proliferation rates of commercial HSCs (MTT assay) in the presence of allogeneic hWJSCs (hWJSC-BM and hWJSC-EM) and hWJSC-CM (BCM and ECM) after 9 days of culture. B: Mean ± SEM live (blue) and $CD34^+$ (yellow) cell counts of commercial HSCs in the presence of allogeneic hWJSC-BM and BCM after 9 days of culture. C: Mean ± SEM live (purple) and $CD34^+$ (yellow) cell counts of commercial HSCs in the presence of allogeneic hWJSC-EM and ECM after 9 days of culture. D: Contour maps of FACS analysis of commercial $CD34^+$ HSCs when they were cultured in the presence of allogeneic hWJSCs and hWJSC-CM. a: ECM, (b) hWJSC-EM, (c) EM, (d) BCM, (e) hWJSC-BM, and (f) BM. Each contour map represents the percentage of $FITC^+$ cells against unstained controls for $CD34^+$ markers. White peak: isotype control; green peak: experimental sample. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

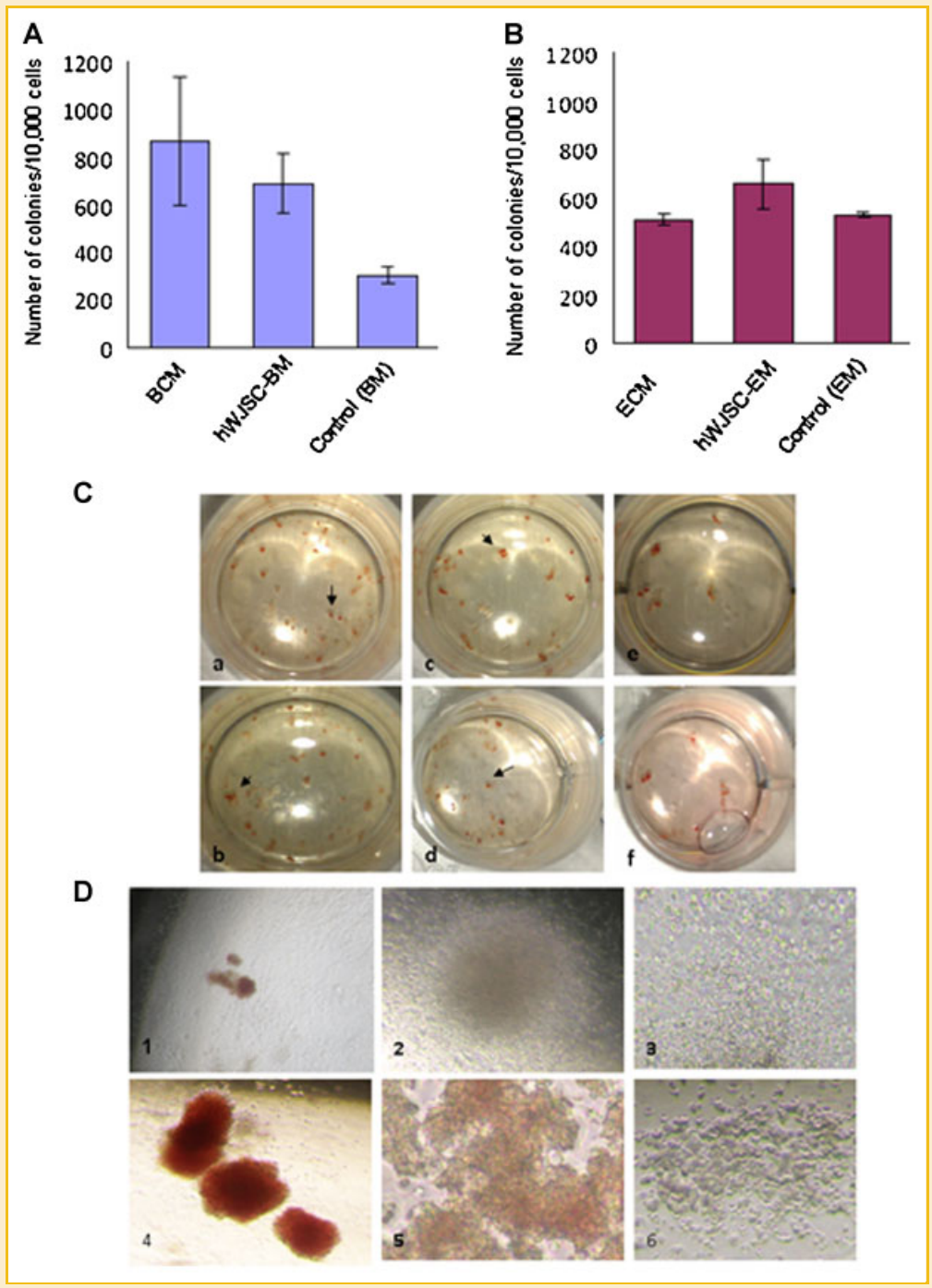

Fig. 3. A: Mean ± SEM colony numbers (CFU assay) of commercial CD34$^+$ HSCs when cultured in the presence of hWJSC-BM and BCM. B: Mean ± SEM colony numbers (CFU assay) of commercial CD34$^+$ HSCs when cultured in the presence of allogeneic hWJSC-EM and ECM. C: Commercial CD34$^+$ HSC colonies growing in duplicate wells of 24-well plates. a,b: BCM; (c,d) hWJSC-BM; and (e,f) BM (control). Note greater number of colonies in BCM. D: Six different types of colony morphology observed with commercial HSCs cultured in the presence of allogeneic BCM: (1) erythroid colony forming unit (CFU-E), (2) granulocyte colony forming unit (CFU-G), (3) granulocyte/macrophage colony forming unit (CFU-GM), (4) erythroid burst forming units (BFU-E), (5) macrophage colony forming unit (CFU-M), and (6) granulocyte–erythrocyte–macrophage–megakaryocyte colony forming unit (CFU-GEMM). [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

high compared to controls (IL-6: 203.82 ± 6.78 vs. 0.16 ± 0.01; IL-8: 1875.94 ± 74.33 vs. 4.72 ± 0.14; HGF: 2272.81 ± 5.65 vs. 409.05 ± 29.06; SCF: 16,390.19 ± 0.15 vs. 4.74 ± 0.02; ICAM-1: 118.86 ± 2.09 vs. 0 or less than detectable range).

DISCUSSION

Since HSCs can differentiate into the full spectrum of mature blood cells they have been used clinically for the treatment of malignant

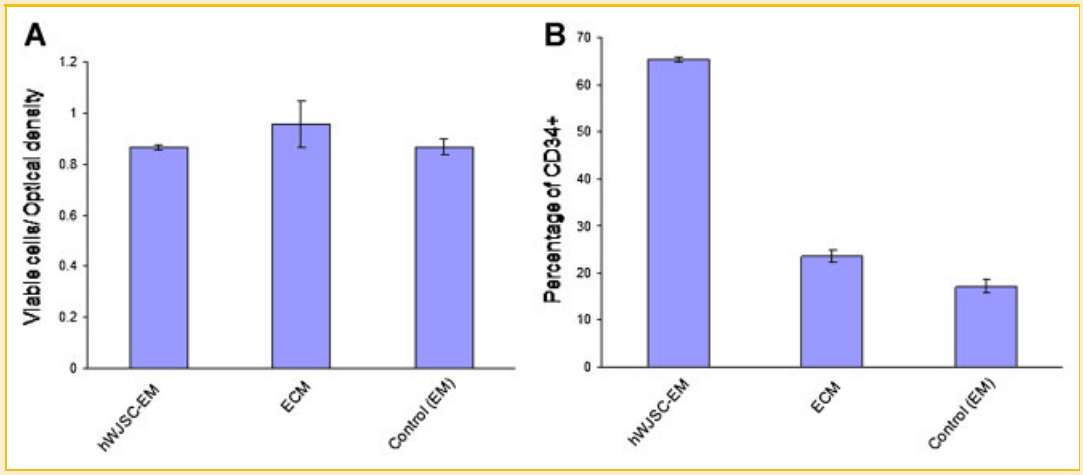

Fig. 4. A: Mean ± SEM proliferation rates of HSCs (MTT assay) in the presence of autologous hWJSC-EM and ECM after 9 days of culture. B: Mean ± SEM $CD34^{+}$ HSC percentages after FACS analysis when cultured in the presence of autologous hWJSC-EM and ECM after 9 days of culture. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

and non-malignant diseases of the hematopoietic system. Unfortunately, however, bone marrow HSCs have the disadvantages of insufficient numbers, painful harvest, potential morbidity, and risk of infection. The low HSC numbers present in UCB together with their slow self-renewal in vitro has limited their use to only children and not adults. Two therapeutic strategies have therefore been explored by cord blood banks to help increase HSC numbers for treatment. These include (1) double cord blood transplant (CBT) and (2) ex vivo expansion [Robinson et al., 2011]. Double CBT significantly delayed engraftment and increased engraftment failure and only one of the CBT units predominated during engraftment [Robinson et al., 2011]. It was shown that increasing the dose of the cell subpopulations responsible for rapid engraftment improves neutrophil and platelet counts for engraftment and reduces engraftment failure. This therefore was the rationale behind the need for the development of reliable ex vivo HSC expansion methods [Robinson et al., 2011].

A variety of stromal cells have been studied as matrices for ex vivo expansion of HSCs and these include in-house derived primary cell monolayers [Breems et al., 1998], genetically modified cytokine-releasing cells [Balduini et al., 1998], bone marrow MSCs (BMMSCs) [Noort et al., 2002], and immortalized BMMSCs [Nishioka et al., 2003]. The mechanism of action of such feeder-cell matrices was the production of a sustained release of secretory molecules that promote HSC self-renewal and maintenance [Zhang et al., 2006]. Fresh patient-aspirated or commercial BMMSCs thus

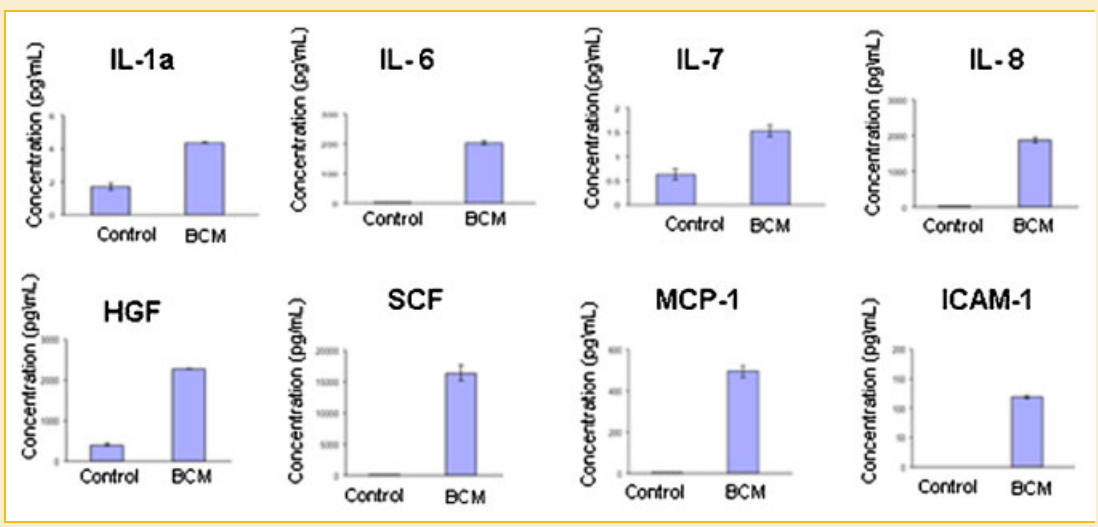

Fig. 5. Proteomic analysis of BCM showing significantly greater mean ± SEM levels (pg/ml) of IL-1a, IL-6, IL-7, IL-8, HGF, SCF, MCP-1, and ICAM-1 compared to controls. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

far have been the cell of choice for stromal support of HSCs on the premise that such MSCs are normally resident in the bone marrow and their natural in vivo role was to provide a scaffold for the expansion of neighbouring HSCs. However, because of the pain and risk of infection in harvesting fresh BMMSCs and the variability and cost in using commercial BMMSCs the use of UCB-MSCs have been explored for ex vivo HSC expansion. Unfortunately, UCB-MSCs also have their own limitations in that some groups were successful in isolating MSCs from UCB [Erices et al., 2000; Goodwin et al., 2001; Bieback et al., 2004; Lee et al., 2004] while others failed or obtained very low MSC numbers [Mareschi et al., 2001; Romanov et al., 2003; Wexler et al., 2003]. In fact only 29 MSC-like colonies were isolated in 17 of 59 processed cord blood units with optimal cell growth being reached as late as 20 days in culture [Bieback et al., 2004].

hWJSCs or hWJSC-CM is available in abundance and is an attractive source of allogeneic or autologous support for ex vivo HSC expansion. Using the protocol described in the present study the derivation efficiency for hWJSCs was 100% (hWJSCs were harvested and successfully propagated in all of 12 different patients) and hWJSC numbers of approximately $4–5 \times 10^6$ cells/cm of umbilical cord were consistently obtained. Such MSC numbers without serial culture are very much higher than what is available in bone marrow or UCB.

Time lapse imaging, phase contrast optics, and SEM showed that in the presence of hWJSCs or its hWJSC-CM the HSCs began to put our pseudopodia-like outgrowths to help them migrate towards the surface of the stromal hWJSCs looking for niches to attach and proliferate. Such outgrowths have been previously described as ''fleet feet'' [Frimberger et al., 2001] or ''uropods'' [Alakel et al., 2009]. Rapid motility and directed migration towards stromal cells with membrane modulation were also observed using high-speed optical sectioning microscopy and inverted fluorescent video microscopy [Frimberger et al., 2001]. In fact, Alakel et al. [2009] showed that direct contact of HSCs with MSCs affected migratory behavior and gene expression profiles of $CD133^+$ HSCs during ex vivo expansion. Additionally, it was postulated that specific stem cell niches within the stromal cell monolayers played a role in HSC migration and proliferation and the MSC surface was the predominant site of HSC proliferation while the compartments beneath the MSC layer mimicked the stem cell niche for more immature cells [Jing et al., 2010].

hWJSCs have been shown by many workers to secrete a wide variety of factors including members of the interleukin family, glycosaminoglycans (GAGs), hyaluronic acid (HA), cell membrane proteins, cell adhesion molecules, cadherins, and growth factors [Friedman et al., 2007; Angelucci et al., 2010]. The results of proteomic analysis in the present study are consistent with these reports with levels of most of these proteins being much higher than that observed in BMMSC and other stromal cell types. The members of the interleukin family specifically IL-6 and IL-8 and the growth factors (SCF, HGF) may be the important players in bringing about the ex vivo HSC expansion as their values in the BCM in this study were extremely high. IL-6 has been reported to stimulate hematopoiesis [Patchen et al., 1991] and IL-8 enhances the proliferation of $CD34^+$ cells [Corre et al., 1999] while SCF and HGF were shown to be effective in HPC maintenance [Sell, 2004]. Additionally, hWJSCs naturally secrete high levels of HA and GAGs which are the building blocks of the extracellular matrix [Fong et al., 2011]. Interestingly, it was recently shown that when heparan sulfate (a member of the GAGs family) was administered alone to $CD34^+$ cells it helped to expand and maintain the morphology of blood lineages in vitro [Bramono et al., 2011].

The hWJSC-CM in the present study provided as good or better support than the cells themselves for HSC expansion. This would be more appealing to regulatory bodies for clinical application as hWJSC-CM is a non-cellular liquid, defined, safer and excludes the potential transmission of adventitious agents from stromal supporting cells to the HSCs. Bhatia et al. [1997] reported the expansion of long term culture-initiating cells (LTC-ICs) and colony forming cells (CFC) when they used a bone marrow stromal-conditioned medium supplemented with IL3 and macrophage inflammatory protein 1 (MIP-1a). hWJSC-CM is not only naturally rich in these specific agents reported by Bhatia et al. [1997] but possess additional useful interleukins (IL-1a, IL-6, IL-7, and IL-8) and growth factors and as such would not need any supplementation to bring about even greater desirable effects.

The logistical limitations of using BMMSCs from a family member of the patient as stromal support for ex vivo expansion of HSCs at the time of treatment has been highlighted [Robinson et al., 2011]. These limitations included (1) the appropriate family member not always being available to donate the bone marrow and (2) and the long time taken to not only generate sufficient BMMSCs (3 weeks) but also carry out coculture expansion (2 weeks) to provide adequate expanded HSCs (5 weeks) since the disease progression was very rapid in some leukemic patients. These workers therefore recommended the availability of a source of ''off-the-shelf'' GMP-compliant allogeneic MSCs for immediate use to alleviate this logistic problem. The results of the present study endorses these suggestions and recommends that allogeneic hWJSCs could be an attractive source of such ''off-the-shelf'' MSCs.

The results of the present study also demonstrated that autologous hWJSCs harvested from the same patient's umbilical cord also provided a useful source of stromal support cells for $CD34^+$ expansion. This would also be ideal as there would be in no immunogenic issues when it comes to cell matching even though allogeneic hWJSCs in general are considered hypoimmunogenic [Weiss et al., 2008]. Harvesting of hWJSCs takes only a few hours [Fong et al., 2007, 2010] and from a practical point of view, it is possible to freeze and store UCB-HSCs and hWJSCs of the same patient on the same day in a dual chamber blood collection bag. When the HSCs are needed, the hWJSCs could be thawed, confluent monolayers established to generate 24 h hWJSC-CM which can then be used for the expansion of HSCs when their numbers are low. This approach will avoid the need for discarding HSC samples that are low in numbers as is practiced by cord blood banks today.

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