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Wharton's Jelly Mesenchymal Stromal Cells as a Feeder Layer for the Ex Vivo Expansion of Hematopoietic Stem and Progenitor Cells: a Review

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Abstract In recent years, umbilical cord blood (UCB) has been widely used as an alternative source to bone marrow (BM) for transplantation of hematopoietic stem and progenitor cells (HSPCs) in a variety of hematological and non-hematological disorders. Nevertheless, the insufficient number of UCB-HSPCs for graft represents a major challenge. HSPCs ex vivo expansion prior to transplantation is a valid strategy to overcome this limit. Several attempts to optimize the expansion conditions have been reported, including the use of mesenchymal stromal cells (MSCs) as feeder layer. Wharton's Jelly (WJ), the main component of umbilical cord (UC) matrix, is especially rich in MSCs, which are considered ideal candidates for feeder layer in co-culture systems. In fact, they can be easily harvested and grow robustly in culture, producing a confluent monolayer in a short time. Similarly to bone marrow-mesenchymal stromal cells (BM-MSCs), WJ-derived MSCs (WJ-MSCs) have been used to support hematopoiesis in vitro and in vivo. Here, we review the rationale for using MSCs, particularly WJ-MSCs, as a feeder layer for UCB-HSPCs ex vivo expansion. In addition, we report the main findings attesting the use of these MSCs as a support in hematopoiesis.

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Introduction

The umbilical cord (UC) is an extra-embryonic formation which links the developing fetus to the placenta. Recently it has emerged that the tissues within the UC are a reliable source of both hematopoietic stem/progenitor cells (HSPCs), derived from umbilical cord blood (UCB), and mesenchymal stromal cells (MSCs), derived from the perivascular tissues. The UC is a perinatal tissue, easily accessible with no risks for the donor and causing no ethical concerns. UCB is considered an alternative source of HSPCs for hematopoietic stem cells transplantation (HSCT) with respect to bone marrow (BM) and peripheral blood, because of the easy availability, tolerance of HLA-mismatch, and low incidence of graft versus host disease (GVHD) [1]. Moreover, UCB-derived HSPCs have a higher frequency of progenitors with greater clonogenic potential compared to their adult counterpart [2, 3]. Unfortunately, the yield of UCB-HSPCs from a single unit is insufficient for the transplant in adult patients. Therefore, several attempts have been made to expand these cells in vitro by using multiple combinations of specific media, cytokines, growth factors and more recently also mesenchymal stromal cells (MSCs) as feeder layers. The rationale behind the use of MSCs as a stromal support in in vitro settings is based on the physiological role of these cells in BM, where they act as a natural scaffold for HSPCs growth. A wealth of evidence exists showing that MSCs influence HSPCs homing, retention, proliferation and differentiation by means of two mechanisms: cell-to-cell contact and paracrine factors secretion [4]. Many preclinical and clinical studies have

demonstrated that the co-transplantation of UCB-HSPCs with BM-MSCs enhances the hematopoietic cell engraftment and accelerates platelet and neutrophil recovery after transplantation [5]. Wharton's jelly-MSCs (WJ-MSCs) isolated from UC-matrix may be an ideal candidate for the stromal feeder layer in co-culture systems, in that they are easily harvested and can readily be expanded to reach a confluent monolayer in a short time [6]. Conversely to BM-MSCs, only a limited number of studies have investigated the role of WJ-MSCs in supporting ex vivo expansion of HSPCs. In this review, we discuss the rationale for using MSCs, in particular WJ-MSCs, as a feeder layer in HSPCs ex vivo expansion and we report the main results obtained in this field.

Human Umbilical Cord Tissue: a Source of Mesenchymal Stromal Cells and Hematopoietic Cells

The umbilical cord is essential for fetal development since it contains the umbilical vessels that provide the blood flow between the developing fetus and the placenta during pregnancy. The histological organization of human UC reveals the presence of multiple tissue layers. Externally the UC consists of an umbilical epithelium, also known as amniotic epithelium. Beneath which, a thick layer of mucous connective tissue, named Wharton's jelly (WJ), embeds the umbilical vessels (two arteries and one vein). The main physiological function of Wharton's Jelly is to prevent compression, torsion and bending of the vessels during pregnancy. WJ contains an abundant extracellular matrix which is particularly rich in glycosaminoglycans (GAGs), mainly hyaluronic acid (HA) and proteoglycans. The fibrillar component is less abundant and contains several types of collagen molecules of either a fibril- or non-fibril-forming types. The cells residing in WJ have been the object of studies in past years, and were classically identified as either myofibroblasts or atypical fibroblasts [reviewed in 7]. Several authors have demonstrated that the most of cells contained in the stromal compartment exhibit the vast majority of features typical of mesenchymal stromal cells [7, 8]. UCB is a hematopoietic tissue which contains a heterogeneous population of hematopoietic cells, either stem or progenitor cells capable of self-renewal and of generating all blood cell types, respectively [9]. Phenotypically, HSPCs are identified by the expression of CD34 surface glycophosphoprotein, [10] and by the absence of all lineage specific markers (Lin negative). In UCB, this primitive population represents about 0.1–0.4 % of total nucleated cells (TNCs). Within the Lin−/CD34+ cell population, the CD38 marker is used to differentiate multipotent progenitors (CD38-), and committed progenitors (CD38+). The subpopulation Lin−/CD34+/CD38- represents about 10 % of HSPCs in UCB [11, 12]. The co-expression of CD90 or CD133 on CD34+/CD38- cells defines the hematopoietic stem cells with long-term repopulating ability [12, 13]. Compared to adult HSPCs, UCB-derived HSPCs are easily available at birth and have higher in vitro proliferation capacity with superior in vitro clonogenic potency and in vivo engraftment capacity, and therefore they can enhance BM-reconstitution after transplantation [14–16]. These features make UCB grafts as elective choice for HSCT in patients with hematological diseases and other disorders such as lysosomal storage disorders (LSD) [17]. Unfortunately, the yield of UCB-HSPCs from a single unit is often insufficient for transplant in an adult patient. Therefore, different strategies have been employed to increase the number of UCB-HSPCs while maintaining their repopulating capacity. In this regard, the feeder co-culture system with mesenchymal stromal cells, derived from bone marrow or perinatal tissues, represents an optimal approach to overcome this limit.

WJ-MSCs: Main Features and Mechanisms of Interactions with Hematopoietic Cells

WJ-MSCs fulfill all the criteria proposed by the International Society for Cellular Therapy (ISCT) to define a MSC population: expression of markers, cellular morphology, multipotency, ability to self-renew, and plastic adherence [18]. WJ-MSCs have some traits in common and some distinctive traits with respect to BM-MSCs [6, 19]. Due to their relative "youth", WJ-MSCs, as well as other perinatal cells, have a higher expansion ability with faster population doubling time than the adult MSCs. The expansion capability is linked to a high telomerase activity typical of stem cells [20]. WJ-MSCs are considered a therapeutic tool for regenerative medicine applications thanks to their ability to trans-differentiate into multiple mature cell types in vitro and in vivo [20–29]. Apart from the classical markers that identify the MSC population according to ISCT criteria (CD73, CD90, CD105), a few recent papers have reported other markers expressed by WJ-MSCs and BM-MSCs. Surface antigens such as CD10, CD13, CD44, CD49e, and CD166 are now recognized as part of their phenotype [20, 27–34] (Table 1). Intriguingly, recent papers have also highlighted that WJ-MSCs express several other molecules which may further help to characterize their phenotype in vitro and elucidate some of the possible interaction pathways with HSPCs and/or mature blood cells. For example, CD117 (c-kit), the receptor for the stem cells factor (SCF) harboured by HSPCs, has been repeatedly detected in WJ-MSCs and little is known about the role of this interaction [27]. Nilsson and co-workers were the first to show that WJ-MSCs express the osteopontin gene [35], while Raio et al. found that WJ-MSCs are able to secrete hyaluronic acid [36]. Interestingly, both these molecules are among the main constituents of the HSPCs niche. In particular, osteopontin is a critical regulator of HSPCs localization and proliferation [35]. WJ-MSCs as well as other MSCs

Table 1 Comparison of constitutive markers and molecules involved in HSPCs expansion, expressed in perinatal (WJ-MSCs) and adult (BM-MSCs) mesenchymal stromal cells

	WJ-MSCs	BM-MSCs
Mesenchymal markers		
CD10	+	+
CD13	+	+
CD29	+	+
CD44	+	+
CD49e	+	+
CD73	+	+
CD90	+	+
CD105	+	+
CD166	+	+
Hematopoietic/ endothelial/monocitic markers		
CD11b	−	−
CD14	−	−
CD31	−	−
CD33	−	−
CD34	−	−
CD45	−	−
Stem cells specific tissue factors		
Oct3/4A	+	+
Nanog	+	+
Sox2	+	−
ABC transporters		
ABCG2	+	+
MDR3	+	+
Molecules involved in the HSPC expansion/interaction		
Flt-3	+	+
G-CSF	+	−
GM-CSF	+	−
IL-6	+	+
M-CSF	+	+
SCF	+	+
SDF-1	+	+
CD117	+	−
Immunomodulatory molecules		
B7–1 (CD80)	+/−	−
B7–2 (CD86)	−	−
HLA-ABC	+	+
HLA-DR	−	−
HLA-E	+	+
HLA-F	+	**n.a.**
HLA-G	+	+

MDR-3 multidrug-resistence-3, *Flt-3* Fms related tyrosine kinase-3, *G-CSF* granulocyte-colony stimulating factor, *GM-CSF* granulocyte-macrophage- colony stimulating factors, *M-CSF* macrophage-colony stimulating factor, *SCF* stem cell factor, *SDF-1* stromal-derived factor-1

express CXCL12 (CXC-Chemochine-ligand-12, also known as SDF-1α, stromal derived factor-1), an important regulator of hematopoiesis and HSPCs homing. This chemokine, by interacting with its receptor CXCR4 (CXC-Chemokine-receptor 4) localized on HSPCs surface, plays a role in the cyclical HSPCs mobilization and homing from and to the BM vascular niche [37–39]. Most of the cytokines involved in hematopoiesis regulation known to be expressed by BM-MSCs are also secreted by WJ-MSCs (see Table 1). Key examples are interleukin (IL)-6, SCF, Fms-related-tyrosine kinase-3 ligand (Flt-3 ligand), and macrophage-colony stimulating factor (M-CSF). Conversely, unique to WJ-MSCs is the ability to secrete further molecules associated with HSPC proliferation, such as granulocyte-colony stimulating factor (G-CSF) and granulocyte-macrophage -colony stimulating factor (GM-CSF) [40, 41]. All this evidence supports the idea of using these cells as feeder layers for HSPCs expansion. With the growing number of reports adding new information on WJ-MSCs secretome and basic biology, it can be expected that their ability to interact with HSPCs will be further confirmed in the near future. Moreover, further research needs to be done in order to clarify the role of WJ-MSCs in the microenvironment in supporting fetal hematopoiesis, and confirm the use of these cells as feeder layers for the expansion of hematopoietic progenitors. In addition to the hematopoietic-supportive function, MSCs are used in transplantation settings to constrain the immune response of recipient to donor cells, thus limiting graft rejection [42]. MSCs exert this immunosuppressive function via modulation of both cellular and innate immune pathways [43, 44]. WJ-MSCs derive from a naturally immune-privileged tissue and, by maintaining a positional memory of their features, may have a selective advantage to escape the immune system also in an immunocompetent environment, more than their adult counterpart [45]. Extended characterization of WJ-MSCs immune molecules has revealed the expression of non-classical class Ib MHC molecules such as HLA-G (both the membrane-located molecule and its soluble form HLA-G5), HLA-F, HLA-E, together with other molecules, such as the Early Pregnancy Factor (EPF) which is a secreted molecule currently undergoing investigation [46–49]. All of these factors have a key role in the induction of tolerance of the mother's immune system toward the semi-allogeneic embryo, in particular for maternal NK cells [46]. Several mechanisms have been proposed to explain the hypoimmunogenicity and immune-modulation caused by WJ-MSCs [50–52], however this field needs to be extensively explored in order to improve their clinical application.

Rationale for UCB-HSPCs Ex Vivo Expansion

Unrelated UCB transplantation has become one of the standard therapeutic options for pediatric patients with hematological

malignancies and various genetic, hematological or immunological disorders [reviewed in 53–55]. Compared to BM grafts, the UCB graft offers substantial advantages such as greater availability of banked cryopreserved UCB units, with consequently shorter time to UCBT (a median of 25–36 days earlier compared to BM); a wider donor pool due to tolerance of 1–2 HLA mismatches out of 6 (higher HLA mismatch is associated with lower probability of engraftment); lower risk of transmitting infections by latent viruses; and lower incidence and severity of GVHD without compromising graft versus leukemia/lymphoma (GvL) effects [56]. The lower incidence and severity of GVHD in UCB recipients is the direct consequence of the biological properties displayed by UCB lymphocytes: reduced proliferation, cytokine production, and cytotoxicity to alloantigens [57]. Despite these logistical and clinical advantages, the UCBT in adult patients is limited by the low number of progenitors present in UCB unit. This limit causes a slower engraftment time with consequent slightly higher risk of disease relapse and mortality from bacterial infections due to delayed immune system recovery [56, 57]. To overcome these drawbacks and increase the number of transplantable HSPCs, two main strategies have been investigated: infusion of two UCB units, and ex-vivo expansion of UCB-HSPCs [58, 59]. Although the co-transplantation of two un-manipulated UCB units led to significant improvements over the single unit infusion, the hematopoietic recovery remains often suboptimal [60]; this has been correlated with the observation that only cells from one UCB ultimately predominated in the reconstituted BM [61]. Therefore, recently, efforts have been focused on the development of ex vivo expansion technologies to increase the total number of UCB-HSPCs while preserving their stemness and capacity to differentiate in all blood cell types [62, 63].

UCB-HSPCs Expansion Conditions

Various protocols have been developed to promote UCB-HSPCs ex-vivo expansion by using different media, growth factors and cytokines [reviewed in 63]. Most of them converge on the supplementation of three early hematopoietic cytokines: SCF, thrombopoietin (TPO), and Flt-3, known to be important for the growth and maintenance of hematopoietic cell stemness and pluripotency. Further attempts have been made to optimize HSPCs ex vivo expansion rate with the addition of other cytokines, IL-3, IL-6, IL-11 and G-CSF, known to be involved in the maintenance of primitive progenitors. However, the cytokine-mediated expansion methods generated only moderate increases in the number of progenitor cells with consequently modest improvement in neutrophil and platelet recovery after transplantation [62, 64–66]. For this reason, the need to improve expansion conditions led to the use of other growth factors known to enhance cell proliferation, such as angiopoietin-like proteins (Angptl-protein) [67], pleiotrophin [68] and insulin-like growth factor-binding protein-2 (IGFBP-2) [69, 70]. Apart from peptides and proteins, also small molecules and chemical compounds have also been investigated for their ability to promote progenitor cells expansion [71]. This was the case of tetraethylenepentamine (TEPA, a copper chelator) [72], StemRegenin 1, identified as aryl hydrocarbon receptor antagonist (SR1) [73], and nicotinamide (NAM), a potent inhibitor for both NAD + −dependent enzymes and sirtuin-1-inhibitor [74]. All these molecules enhanced the hematopoietic stem cells proliferation and differentiation. Another chemical compound, UM171, in cooperation with SR1, has been demonstrated to increase the number of long-term hematopoietic progenitors and inhibits differentiation toward erythroid and megakaryocytic lineages [75]. One of the most potent molecules that promotes ex vivo HSPCs amplification is Notch ligand [76] which plays a critical role in self-renewal, survival, proliferation and differentiation of stem and progenitor cells in vitro and in enhancing engraftment in vivo [77]. Since most of these studies confirm that the cytokine-driven expansion conditions are accompanied by concomitant cell differentiation, in recent years the use of stromal cells has been suggested as a more natural approach to augment the number of UCB-derived HSPCs [78].

Rationale of MSCs as a Feeder Layer to Enhance UCB-HSPCs Expansion

The long-term fate of HSPCs depends on migration, adhesion to the niche, and subsequent regulation of self-renewal versus differentiation state [79]. All these properties are regulated by cues provided in vivo by the niche cellular microenvironment. MSCs, together with endosteal cells, vascular cells and pericytes, form the BM niche and play a key role in supporting both maintenance and differentiation of the stem cell pool [80–84]. Therefore, this supportive interaction could be mimicked by an in vitro model where HSPCs are co-cultured in the presence of MSCs as stromal support [85, 86]. Although numerous studies have demonstrated the capacity of MSCs as a feeder to maintain the undifferentiated state of HSPCs with different degrees of efficacy [87–89], little is known about the precise cellular and molecular mechanisms involved in these interactions. It has been suggested that MSCs could support hematopoiesis by two mechanisms: direct cell-to-cell contact and secretion of specific factors [37, 78]. Some reports demonstrated that cell-to-cell contact is crucial to promote expansion of stem cell progenitors [38] and some authors even highlighted after expansion, two hematopoietic cell populations with different phenotypic and biological characteristics: one adherent to the stromal layer and the other floating in the medium [90]. The notion that the contact with MSCs

influences the hematopoietic cell behavior and fate is supported by microscopical observations. The hematopoietic cells form pseudopodia-like protrusions and acquire high motility when co-cultured in the presence of a MSC monolayer [91]. Moreover, in the co-culture system it is possible to observe clusters of tightly packed cells, referred to as a cobblestone areas, composed of hematopoietic progenitors that adhere to the stromal cells [92]; the ability of HSPCs to form cobblestone areas is currently used as an in vitro test, (cobblestone area-forming cells assay, CAFC), to assess the frequency of long-term BM-reconstitution HSCs.

It is not definitely known whether the interaction between MSC and HSPCs, resulting in HSC proliferation, is mediated by diffusible factors crossing over from the MSCs to HSPCs during cell-to cell contact or through secretion of them by the MSCs into the microenvironment where HSPCs are located [91]. Different reports have highlighted that soluble factors released by MSCs are sufficient to promote hematopoietic cells expansion [86, 93]. Among these, some growth factors, chemokines and cytokines such as tumor necrosis factor-alpha (TNF-α), transforming growth factor-beta1 (TGF-β1), leukemia inhibitory factor (LIF), CXCL12, SCF, M-CSF, IL-3, IL-6, TPO and Flt-3 ligand have been implicated in the regulation of either survival, proliferation, homing or differentiation of HSPCs [37]. In particular, elevated levels of CXCL12 in the co-culture system have resulted in down-regulation of a number of cytokines and chemokines that promotes HSPCs differentiation and restrains self-regeneration [38, 94]. The enhanced engraftment reported in in vivo studies after co-transplantation of MSCs and HSPCs is probably related to the production by MSCs of cytokines and growth factors, such as SDF-1α, that promotes the homing and expansion of the transplanted HSPCs [95, 96]. In this context, an extended characterization of the intrinsic properties of stromal cells as feeder will be mandatory to better understand their potentiality as a tool for HSPCs expansion.

BM-MSCs Support Hematopoiesis

The ability of BM-MSCs to support hematopoiesis has been reported in both pre-clinical and clinical studies. The pathways through which the hematopoietic supportive function of stromal cells is exerted are not fully elucidated. Some authors have provided evidence that the direct cell-to-cell contact is crucial to promote HSPCs growth [97, 98] and to preserve their stemness [99], while others suggest that the contact is more essential for the regulation of mature blood cells production [100, 101]. Conversely, some papers pointed out that the secreted paracrine soluble factors are key players in hematopoiesis [84, 102]. Different studies have attempted to establish the optimal combination and concentration of different cytokines to expand HSPC in culture in presence of BM-MSCs, with divergent results [5, 78, 103–106]. The addition of two epigenetic regulators, namely 5-aza-deoxycytidine (aza-D) and trichostatin A (TSA), to the culture medium was found to enhance BM-MSCs feeder action to maintain the UCB-HSPCs stemness [107]. Another strategy to strengthen the BM-MSCs function as feeder layers is their genetic manipulation in order to induce the secretion of crucial factors for HSPCs growth such as angiopoietin-like 5, [108] or the expression of human telomerase reverse transcriptase (hTERT) [109]. More recent studies focused on miming the BM niche in vitro by seeding the MSCs on three-dimensional (3D) collagen or fibrin scaffolds together with HSPCs. An increased migration of HPSCs toward MSCs was observed and, in addition, higher levels of molecules involved in the maintenance of a quiescent pool of HSPCs were detected [110]. Moreover, a synergic action between 3D scaffolds and MSCs to increase the proliferation of HSPCs retaining a more primitive phenotype has been reported [110, 111]. The supportive functions of BM-MSCSs have been proved in mouse models where the co-transplantation of BM-MSCs with UCB-HSPCs resulted in higher hematopoietic engraftment versus UCB-HSPCs transplantation alone [41, 112, 113]. All these successful in vitro and in vivo findings opened the way to clinical translation. De Lima and co-workers transplanted 31 myeloablated patients affected by hematologic malignancies with two UCB units, one un-manipulated and one expanded for 14 days on BM-MSCs layers in the presence of SCF, TPO, Flt-3 ligand and G-CSF. This protocol significantly improved engraftment compared to that of using un-manipulated double UCB units as a graft. However, long-term follow-up showed that only the cells derived from the un-manipulated UCB unit ultimately contributed to long-term donor-derived hematopoiesis. The authors attributed the positive engraftment results to the increased numbers of committed progenitors in the expanded cells that accelerated the hematopoietic recovery [114]. The long time needed to expand a sufficient number of BM-MSCs is unfortunately a logistical problem correlated to the use of these cells as a feeder layer for HSPCs ex vivo expansion, since the progression of some hematological diseases, such as leukemia, sometimes has a fast clinical course. For this reason, MSCs derived from alternative sources, in particular UC-matrix derived MSCs, have been employed for their prompt availability and great proliferation capacity.

State of the Art on the Use of WJ-MSCs as a Feeder Layer for HSPCs Expansion

The amount of recent data coming from laboratories worldwide attests the use of WJ-MSCs as feeders for HSPCs growth and maintenance in vitro and as support for engraftment in vivo. In this regard, the advantages of using WJ-MSCs as

stromal support, alternatively to BM-MSCs, can be summed up in the following key features: immediate availability, painless procedures to donors, lower risk of viral contamination, higher ex vivo expansion rate, and expression of specific cytokines and growth factors that are involved in the hematopoiesis. Several attempts have been made to define the role of WJ-MSCs to support ex vivo HSPCs expansion: the main findings are summarized in Table 2. In 2006, Lu and colleagues [40] were the first to establish a protocol to isolate abundant MSCs from UC tissue by an enzymatic procedure and to show their hematopoiesis supportive function. They found that UC-MSCs shared several phenotypic and molecular features with BM-MSCs but had higher in vitro colony–forming unit-fibroblast (CFU-F) potential and higher proliferation ability as demonstrated by a faster cell population doubling time. The authors highlighted in UC-MSCs the expression of cytokines and growth factors involved in hematopoiesis (SCF, LIF, Flt-3 ligand , IL-6, M-CSF , VEGF) as well as in the HSC engraftment (SDF-1). The levels of expression of these factors were comparable to those to BM-MSCs; additionally, the UC-MSCs also expressed GM-CSF and G-CSF, growth factors crucial to expand granulocyte and macrophage progenitors. The hematopoietic supportive function was assessed by co-culturing CD34+ cells from allogeneic cord blood with UC and BM-derived MSCs as feeder layers. After 5 weeks, the clonogenic potential of both co-cultured CD34+ cells was evaluated in a long-term CFU assay and no difference in the number of CFU-GEMM, BFU-E CFU-GM colonies generated from the most primitive progenitors was reported. In addition, the typical cobblestone areas generated by primitive hematopoietic stem cells were observed in both co-culture systems. Overall, these data highlighted a comparable hematopoietic-supportive potential between UC-and BM-MSCs. In this study, noteworthy was the low expression of the HLA-ABC molecule detected on UC-MSCs in addition to the absence of HLA-DR expression that is typical in MSCs populations. These findings, also reported for human umbilical cord perivascular stromal cells [115], strengthen the usefulness of UC-MSCs for allogeneic cell therapy in that the low expression of the HLA-ABC molecule would limit the host immune response. These promising findings have aroused interest to use UC-MSCs as feeders for ex vivo HSPCs expansion. The potential therapeutic application of WJ-MSCs, as stromal support for the growth and maintenance of UCB-derived hematopoietic stem cells in culture, comes also from the group of Bakhshi [116]. They showed that WJ-MSCs, similarly to BM-MSCs, effectively maintained UCB-CD34+ cells as demonstrated by the capability to form colonies in the long-term culture initiating cells (LTC-IC) assay. Friedman and co-workers [41] reported that UC-MSCs produced significant amounts of hematopoietic cytokines and growth factors and enhanced the hematopoietic engraftment in SCID mice. They observed a greater number of hematopoietic colonies, in particular CFU-GM, when the UCB-mononuclear cells (UCB-MNC) were plated in semisolid medium in the presence of a UC-MSCs monolayer. Moreover, NOD/SCID γcnull mice, when co-transplanted with a limited number of either unfractionated UCB-MNCs or selected UCB-CD34+ cells and UC-MSCs, showed a higher frequency of human CD45+ cells in both bone marrow and peripheral blood compared to mice transplanted with UCB cells alone. The authors suggested that this improvement was due to the capacity of co-injected UC-MSCs to provide an organized stroma essential for the hematopoietic cell engraftment; this function may be sustained by the numerous factors released by UC-MSCs. More recently, Magin and co-workers [117] compared the feeder potential of three primary cell types: BM-MSCs, WJ-MSCs and umbilical cord vein endothelial cells (HUVECs). They found that all these primary cells had a comparable potential to support UCB-CD34+ cells expansion, with WJ-MSCs even superior than the other two cell types. In co-culture systems with UCB and the three cell monolayers, MNC expansion was 30- to 60-fold, colony-forming cells expansion 20- to 40-fold, and cobblestone area-forming expansion 10- to 50-fold. Another study, carried out by Tipnis and co-workers [118], showed that UC-MSCs of autologous or allogenic origin were similarly able to sustain both fresh or cryopreserved UCB-CD34+ cells expansion in the presence of a very low concentration of exogenous cytokines (SCF, Flt-3, TPO, b-FGF). After 14 days of co-culture, both autologous and allogeneic UC-MSCs were able to increase the number of TNCs (about 27-fold), the number of CD34+ cells (24-fold), and the number of colonies developed in short-term CFU assay (more than 5-fold) compared to the uncultured UCB-CD34+ cells. In this study, the expansion potentiality of UC-MSCs of both origins was superior to that of BM-MSCs as a feeder. Even in this study it was ascertained that the UC-MSCs released high levels of SDF-1α, Flt-3, G-CSF, GM-CSF, TPO, angiogenin, HGF, LIF, and IL-6, all of which play an important role in CD34+ cells growth. In addition, the cell adhesion molecules CD29 and CD44, required for homing HSC to BM in vivo, were also expressed. Of note, the CD34+ cells expanded in contact with UC-MSCs became strongly positive for cell adhesion molecules, such as VLA-4 (very late antigen-4) and LFA-1 (lymphocyte function associated antigen-1), known to be involved in the maintenance of stemness. The results reviewed so far, while attesting the hematopoiesis-supportive role of WJ-MSCs, are elusive about the mechanisms underlying this function: cell-to-cell direct contact and/or soluble factors secretion. Fong and colleagues [91] addressed this issue by measuring the proliferation rate and the clonogenic potential of UCB-CD34+ cells when cultured either in direct contact with WJ-MSCs or in WJ-MSCs-conditioned medium (WJ-MSCs-CM) using UCB-CD34+ cells grown alone as control. After 9 days of culture, in a medium supplemented with early hematopoietic cytokines

Table 2 Summary of literature reports indicating the use of WJ-MSCs for the therapy of hematologic diseases or their role in the expansion of HSPCs

MSC population	HSC population	Co-colture scheme	Phenotypic featuresfs of expanded HSCs	Hematopoietic assay	Experiments in vivo	Ref.
Wharton's Jelly Mesenchymal stromal cells (WJ-MSCs) Bone marrow mesenchymal stromal cells (BM-MSCs)	Allogeneic Umbilical cord blood (UCB)derived $CD34^+$ cells	$CD34^+$ cells seeded on irradiated WJ-MSC and BM-MSC layer for 5 weeks, in HCC-5100 medium		I. After co-colture, $CD34^+$ cells were cultured in methylcellulose medium with Il-3, GM-CSF, EPO		[40]
Wharton's Jelly Mesenchymal stromal cells (WJ-MSCs) Bone marrow mesenchymal stromal cells (BM-MSCs)	Umbilical cord blood (UCB) derived MNC Umbilical cord blood (UCB)derived $CD34^+$ cells			I. Hematopoietic colony formation assay. CD34+ cells plated on MSCs or without MSCs	I. UCB cells (unfractioned and CD34+ cells) with UC-MSCs transplanted in NOD/SCID γcnull mice. II. evaluation of HLA CD45 by flow cytometry	[41]
Wharton's Jelly Mesenchymal stromal cells (WJ-MSCs)	Commercial cord blood CD34+ cells	Co-colture of CD34+ cells with: I. autologous/allogeneic WJ-MSCs: 1.in SFEM medium with CC110, 2.without CC110 II. conditioned medium of cultured WJ-MSCs	I. Flow cytometric analysis of CD34+ cells. II.Trypan blue vital CD34 + cells. count. III. CD34+ cells proliferation: MTT assy.	I. Colony forming assay.		[91]
Wharton's Jelly Mesenchymal stromal cells (WJ-MSCs)	Umbilical cord blood (UCB)derived $CD34^+$ cells	WJ-MSCs treated with mitomycin C co-coltured with CD34+ cells in IMDM, FBS, L-glut, Penicillin/streptomycin, hydrocortisone		I. LTC-IC assay: CD34+ cells after co-colture for 35 days with UC-MSC and BM-MSC in methylcellulose with EPO, GM-CSF, IL-3, SCF,		[116]
Bone marrow mesenchymal stromal cells (BM-MSCs) Wharton's Jelly Mesenchymal stromal cells (WJ-MSCs) umbilical cord vein endothelial cells (h -HUVEC)	Umbilical cord blood (UCB)derived $CD34^+$ cells	Co-colture of CD34+ cells with irradiated murine stromal cell lines, BM-MSC, WJ-mSC, HUVEC, in X-vivo 10, with SCF, Flt-3 L, TPO, IL-3 for 7 days		I. CFU- assay II. CFAFC assay II. T- cell activation		[117]
Allogeneic and autologous Umbilical cord matrix-derived mesenchymal stromal cells (UC-MSCs) Allogeneic Bone marrow mesenchymal stromal cells (BM-MSCs)	Fresh and cryopreserved umbilical cord blood derived $CD34^+$ cells	Co-colture for 14 days of fesh and crypreserved UCB-CD34+ cells UC-MSCs and BM-MSCs in: Medium 1: IMDM with 10% , SCF, Flt-3, TPO, bFGF (high concentration of all cytokines) Medium 2: IMDM with 10% , SCF, Flt-3, TPO, bFGF (low concentration of all cytokines)	I. Flow cytometry analysis of CD34, CD33, CD31, CD133, CD38,by cytometer analysis II. karyotipic analysis of expanded Cd34+ cells	CFU assay		[118]
Wharton's Jelly mesenchymal stromal cells (WJ-MSCs)	Umbilical cord blood (UCB)derived $CD34^+$ cells	UCB-CD34+ cells co-cultured	I. Flow cytometry analysis of CD34+ cells. II. flow	I. ALDH assay. II. Clonogenic of HSPCs	I. NOD/SCID mice transplantation: CD34+ cells after 7 and	[119]

Table 2 (continued)

MSC population	HSC population	Co-colture scheme	Phenotypic featuresfs of expanded HSCs	Hematopoietic assay	Experiments in vivo	Ref.
		with irradiated WJ-MSCs and UCB-CD34+ cells in HPO1 serum-free medium for 13 days	cytometry analysis of CD34 + CD38- ; CD34 + CD90+; CD34+ CD33-;CD34 + CD61+; CD34 + CD19+ cell population	assay. III. migration assay of HSPCs	13 days of co-cultured were infused in irradiated NOD/SCID mice. II. evaluation of human CD45+ cells after mice sacrifice at 8 weeks after transplantation	
Wharton' s jelly mesenchymal stromal cells Bone marrow mesenchymal stromal cells (BM-MSCs)	Umbilical cord blood (UCB)derived $CD34^+$ cells	Co-colture for 10 days of irradiated BM-MSC and WJ-MSCs with UCB-cd34+ cells in presence of DMEM with 10% FCS, with TPO	Flow cytometry analysis of CD34 in UCB cells. II. expression of adhesion molecules by expanded CD34+ cells on MSC feeder	I. CFU assay II. Transwell migration assay.	I. NOD SCID mice transplantation: UCB-CD34+ cells alone or in combination with different MSCs were infused in irradiated mice II. evaluation of platelet recovery and human CD45 + cells in peripheral blood II. evaluation of CD34+ cells after co-transplantation with MSCs in BM, blood and spleen	[121]
Bone marrow mesenchymal stromal cells (BM-MSCs) Wharton' s jelly-MSCs Chorion-derived MSCs Amnion-derived MSCs Amniotic fluid-derived MSCs Cord blood-derived MSCs	Umbilical cord blood (UCB)derived $CD34^+$ cells	UCB-CD34+ cell co-coltured for 7 and 14 days, with fresh or cryopreserved MSCs from all sources feeders in α-MEM supplemented with pen/strep, 10% irradiated FBS, G-CSF, SCF, TPO.	Flow cytometry analysis of CD34 and CD61expression in expanded UCB cells	Colony forming unit (CFU-) and long-term culture initiating cell (LTC-IC) assay		[124]
Umbilical cord tissue-MSCs (UC-MSCs) Placental mesenchymal stromal cells (p-MSCs) Bone-marrow mesenchymal stromal cells (BM-MSCs)	Umbilical cord blood (UCB)derived $CD34^+$ cells	Co-colture with irradiated all MSCs in 24 well in presence of Stem pro serum free + IL-6, SCF, TPO, Flt-3 for 10 days	I. Flow cytometry analysis of CD34 , CD38,Cd33, CD19, CD45,CD61,CD3 Ii. Annexin detection after 10 days of co-colture III. evaluation of Wnt and Notch in MSC-HSPCs co-colture by WB. IV. evaluation of cytokines and chemokine present in CM	I. Long-term culture initiating cell (LTC-IC assay. II. CFU assay III. cell-matrix adhesion assay	I. NOD/SCID transplantation: CD34+ cells expanded on UC-MSC and P-MSCs. II. evaluation of short and long engraftment (4th and 12 th weeks after infusion) by evaluation of human CD45+ cells	[126]

HCC-5100 myelocult medium, *GM-CSF* granulocyte-macrophage colony stimulating factor, *IL-3* interleukine-3, *EPO* erythropoietin, *LTC-IC assay* long-term culture- initiating cell assay, *IMDM* Iscove' s modified Dulbecco's medium, *L-glut* L-glutammine, *FBS* fetal bovine serum, *IL-3* interleukine-3, *SCF* stem cell factor, *TPO* trompoiethin, *Flt-3 L* Fms-like tyrosine kinase 3 ligand (CD135), *CAFC assay* Cobblestone area-forming cell assay, *CFU-assay* colony-forming unit assay, *SS, Stem Span* serum-free expansion medium, *CC110* Cocktail of cytokines for expansion, *ALDH assay* aldehyde-dehydrogenase, *HSPC* hematopoietic stem progenitors cells, *WB* western Blotting, *α-MEM* α-minimum essential medium, *G-CSF* granulocyte- colony stimulating factor, *CM* conditioned medium, *CBT* cord blood transplantation

cocktail (SCF, TPO, Flt-3 ligand), a significant increase of CD34+ cells number and frequency was observed in both conditions, with WJ-MSC-CM condition even superior. Moreover, a more than 2-fold increase of colony numbers was found with respect to controls in the CFU-assay, when CD34+ cells were cultured in direct contact with MSCs or in the presence of conditioned medium. All the six morphological colony types were observed in all conditions suggesting a normal hematopoiesis process in vitro. These findings strengthen the use of conditioned medium, either unfractionated or pre-fractionated, for future clinical applications, avoiding the possible side effects of harmful agents transmission from the stromal cells used as support for cell expansion. Interestingly, the authors observed that UCB-CD34+ cells, either in co-culture system or in the presence of CM, presented pseudopodia-like outgrowths and acquired higher motility. Time-lapse microscopy showed that these cellular protrusions helped the UCB-cells to migrate toward the MSC surface, suggesting that MSCs may favor HSPC homing to BM niche. Proteomic analysis of the WJ-MSC-CM revealed high levels of interleukins (IL-1a, IL-6, IL-7, IL-8), as well as SCF, HGF and ICAM-1, suggesting once again that they may be the agents involved in the UCB-CD34+ cells expansion. In a recent work, Milazzo and co-workers [119] proved the capability of WJ-MSCs to expand UCB-CD34+ cells in vitro and to improve their engraftment in NOD/SCID mice. The co-culture of purified-CD34+ cells with WJ-MSCs, without cytokines addition, increased the number of CD34+ cells by 2.0 and 7.0 times after 7 and 13 days respectively, compared to control (uncultured CD34+ cells at day 0). The authors observed, after 7 days of exposure to WJ-MSCs, the expansion of primitive hematopoietic progenitors (CD34+/CD90+ cells, 1.8-fold expansion, and CD34+/ CD33-, 27.4-fold expansion) as well as multipotent early progenitors (CD34+/CD38+, 4.1-fold expansion). The ability by WJ-MSCs to support early hematopoietic progenitors during co-culture was also demonstrated by the amplification of CD34+ cells with higher clonogenic potential (2.2 and 7.5 fold increase after 7 and 13 days, respectively) and higher frequency of ALDH+ cells (enzyme aldehyde dehydrogenase positive cells), in comparison to controls. ALDH is a stem cell marker involved in stem cells differentiation and proliferation [120]. Moreover, cells co-cultured for 7 days on WJ-MSCs migrated more efficiently (30 % higher migratory capacity) compared to CD34+ uncultured cells, in a trans-well migratory assay toward the stromal derived-factor 1α (SDF-1α). The efficacy of the WJ-MSCs to enhance the repopulating capacity of hematopoietic progenitors was evaluated. The expanded CD34+ cells, particularly at 7 days of co-culture, engrafted NOD/SCID mice more efficiently than the uncultured CD34+ cells (100 % versus 75 % transplantation success). The ability of WJ-MSCs and BM-MSCs to enhance engraftment of UCB-CD34+ cell transplanted in NOD/SCID mice was also evaluated by Van De Garde and co-workers [121]. They found that both MSCs populations promoted comparable enhanced recovery of human platelets and human CD45+ cells in mice peripheral blood and found 6-weeks after transplantation a 3-fold higher engraftment of human HSPCs in the BM, blood and spleen when compared to uncultured CD34+ cells. The authors also investigated the phenotypic and functional properties acquired by the UCB-CD34+ cells after direct contact for 10 days with the stromal cells, in the presence of the cytokine TPO. A significant increase of CD49d and CD49e expression was observed in addition to a slight rise of other adhesion molecules (CD11a, CD11b, CD184) associated with homing to or retention in the BM. No increase in the number of TNCs and CD34+ cells or changes in the ratio between different cell subpopulations were reported after co-culture with both WJ-and BM-MSCs. The authors suggested that the culture conditions used, absence of early hematopoietic cytokines and presence of only TPO, may explain the lack of TNCs and CD34+ cells expansion. These results are in agreement with TPO functioning to enhance the platelet repopulation in vivo and to retain the hematopoietic engraftment capacity in NOD/SCID mice [122, 123]. Interestingly, the HSPCs cultured on BM-MSCs stroma were able to generate more BFU-E colonies, while those cultured on WJ-MSCs gave rise to higher numbers of CFU-GEMM generated from the most primitive hematopoietic progenitors. In these studies, the immunosuppressive properties of both MSC populations were confirmed in experiments of T-cell proliferation inhibition of PBMC (peripheral blood mononuclear cells) stimulated with αCD3αCD28 beads. Klein and co-workers [124] compared the hematopoietic ability of WJ-MSCs with other MSCs derived from perinatal tissues including amniotic fluid, amnion, chorion, and cord blood, which were tested for the same tasks. They measured the expansion rates of CD34+ cells after co-culture of UCB-MNCs with MSCs feeders derived from all the above-mentioned perinatal sources, including WJ-MSCs and BM-MSCs. In particular, the fold increase of CD34+ and CD45+ cells was measured after 14 days of co-culture and the clonogenic potential of the cultured cells was determined in short-and long-term assays. The results showed in all the experiments comparable expansion rates of CD34+ and CD45+ cells, and high ability to form CFUs in the short-term assay, particularly when a low number of CD34+ cells (5×10^2 to 1×10^4) were cultured per cm^2 of MSC-monolayer. Expansion of LTC-IC was partly detected in this experiment. However, the expansion of CD34+ cells was significantly more effective when co-cultured with MSCs from BM and amniotic fluid, compared to that observed in amnion, chorion and Wharton's Jelly as feeders. In order to explain the very high expansion rate detected the authors revisited other data in the literature and found similar results when a low number of CD34+ cells and a high concentration of TPO were used. In particular, this cytokine is known to have an anti-apoptotic

effect on HSPCs [125]. Of note, this work also reported that cryopreserved MSCs, compared with fresh ones, had a better capability to expand UCB-CD34+ cells. This result is important in a clinically-oriented interpretation of the data since banked cells are prompt to be employed for therapeutic approaches [124]. The ability of different MSCs to support UCB-CD34+ cells was also investigated by Kadekar and colleagues [126]. They performed a systematic comparison of placental-and cord-derived MSCs characteristics and found that these two cell types presented very a similar morphology, phenotype and potentiality to differentiate toward three cell lineages (osteoblasts, adipocytes and chondrocytes) but had differential ability to support the ex vivo expansion of UCB-CD34+ cells. In particular, placenta derived-MSCs (P-MSCs) resulted to be a better feeder similarly to BM-MSCs for ex vivo maintenance of primitive HSCs, with a higher engraftment potential than that of cells expanded on UC-MSCs layer. Precisely, CD34 + cells expanded on P-MSCs showed a higher percentage of primitive CD34+/CD38-cells, higher CFU and LTC-IC ability and better in vitro adhesion to fibronectin and migration ability toward SDF-1α. The NOD/SCID repopulation ability of HSPCs cultured in the presence of P-MSCs was enhanced compared to that of cells grown on UC-MSCs. After 10 days of co-culture, the authors found a significant 400-fold and 250-fold increase in the number of TNCs when UCB-CD34+ cells were in the presence, respectively, of P-MSCs and UC-MSCs, compared to the uncultured CD34+ cells. The massive expansion reported in the cells co-cultured with P-MSCs did not hinder the quiescent state of the expanded CD34+ cells, as revealed by the high percentage of cells found in G0/G1phase of cell cycle at the end of culture. A further 2.5-fold increase in immature hematopoietic progenitors cells CD34+/CD38-, a higher proportion of the most primitive CD34+/CD133+ cells, and a higher clonogenic potential was reported for CD34+ cells expanded on the P-MSC layer compared to UC-MSCs ones. In particular, there were 1.5 times more CFUs, a 4-fold increase in BFU-E and a 1.6-fold increase in CFU-GEMM. The in vivo studies demonstrated that CD34+ cells expanded in both feeder layers resulted in multi-lineage engraftment in NOD-SCID mice, with enhanced repopulation ability by CD34+ cells expanded on P-MSCs. These functional data correlated well with the different expression of molecules involved in stem cells self-renewal and maintenance by the two mesenchymal cell types. In particular, the authors argued that P-MSCs supported the expansion of functionally superior HSCs, with higher levels of Notch, Wnt and hepatic growth factor (HGF) expression. On the other hand, UC-MSCs facilitated more the expansion of committed progenitors by the secretion of pro-inflammatory cytokines, such as IL-1α, β, human monocyte chemotactic protein2 and 3 (MCP 2,3) and C-C motif chemokine 20 (CCL20, also known as MIP-3α), that drive HSCs differentiation processes [126].

Clinical Application of UC-MSCs and HSPCs Co-Transplantation

Up to now no clinical application of expanded UCB on UC-MSCs feeder layer has been documented. Nevertheless, the promising pre-clinical results of hematopoietic supportive function by UC-MSCs, here reviewed, have led researchers to co-infuse UC-MSCs with UCB-HSPCs to improve hematopoietic cells transplantation outcome. A pilot study carried out by Wu and co-workers [128] evaluated the safety and efficacy of co-transplantation of UC-MSCs with unmanipulated UCB unit. Five pediatric patients, three with non-malignant hematologic diseases and two with leukemia, received the co-transplant, while nine patients were engrafted with UCB alone. The intravenous co-infusion was feasible and no graft failure was reported. All 14 patients reached 100 % cellular chimerism on the 60th day of transplantation. The time needed to achieve neutrophil and platelet recovery was shorter in patients receiving the co-transplant compared to those receiving UCB alone. No transplant–related mortality was reported among the five patients receiving the co-transplant. By contrast, two of the nine patients receiving UCBT alone died for bacterial infections as result of prolonged neutropenia. The same authors reported analogous results in another study of UCBT in 32 patients with high risk of acute lymphoblastic leukemia in which 20 were co-transplanted with UC-MSCs and 12 transplanted with UCB alone [129].

In another study, Wu and colleagues co-transplanted UC-MSCs with adult haploidentical-HSPCs, derived from BM and mobilized peripheral blood, in patients with refractory/relapsed hematological malignancy They reported a fast hematopoietic engraftment and no adverse infusion-related reactions [130]. The same engraftment improvement in patients with severe aplastic anemia (SAA) was reported in other two studies of co-transplantation of UC-MSCs with HSPCs derived from adult sources [131, 132]. The concomitant infusion of MSCs and adult HSPCs seems to be particularly effective in patients with SAA since these patients have defective MSCs that compromise the engraftment of the allogeneic transplanted HSPCs [133, 134]. In this setting, the infusion of normal MSCs may play an important role in providing the specialized BM microenvironment to allow HSPCs engraftment.

Conclusions and Future Perspectives

MSCs play an important role in modulating the BM microenvironment and supporting hematopoiesis. The hematopoietic environment of patients who receive HSCT is often damaged by chemotherapy, irradiation or by the malignant hematological disease per se [135, 136]. For this reason, as well as to induce immune tolerance, the co-transplantation of BM-

MSCs with HSPCs is widely used to reconstitute the damaged stroma and to provide all the hematopoietic cytokines ligands and stem cell factors needed to promote HSPCs homing and engraftment [137, 138]. The co-infusion with MSCs, including WJ-MSCs, at the time of HSPCs has effectively been of benefit in HSCT, in particular in UCBT where the limited number of hematopoietic cells is associated with high graft failure.

As regards their supportive function, MSCs have been exploited as a feeder layer in the co-culture system to increase the number of UCB-derived HSPCs. MSCs isolated from Wharton Jelly appear to offer greater clinical utility compared to BM-MSCs, due to their higher efficiency of recovery coupled with a painless isolation procedure, minimal ethical concerns, higher rate of expansion and lower immunogenicity. A further intriguing feature is their origin from a perinatal tissue. On one hand, this enables WJ-MSCs to interact with hematopoietic and immune cells by means of both cell-cell contact and secetion of paracrine factors. On the other hand, it may be supposed that the common anatomical localization of WJ-and UCB-HSPCs should favor a more efficient cross-talk between the two cell types. Considering the striking differences between the bone marrow stem cells niche and the Wharton's jelly one, more research is needed in this field to ascertain the multiple unknown in vivo roles of WJ-MSCs.

The results of pre-clinical studies reported here attest that the WJ-MSCs are optimal feeders for ex vivo expansion of UCB-HSPCs. The co-culture strategy is more attractive than the cytokine-driven culture condition which still has some constraints that delay the clinical translation: (i) the difficulty to establish the optimal culture conditions to expand and maintain stemness of hematopoietic cells, (ii) the expensive cost of cytokines for large-scale culture and the heterogeneous potency between commercial lots of active culture compounds, and (iii) the cytotoxicity due to the use of non-physiological concentrations. The use of UC-MSCs as a feeder layer overcomes most of the limits of cytokine-driven culture systems. On the other hand, the co-culture condition needs to be also worked out to improve its effectiveness and safety. In particular, protocols of UC-MSCs expansion have to be well defined as regards the type of culture media, the number of cell passages, and the risk of pathogens transmission from cell-to-cell. Moreover, the mechanisms underlying hematopoietic supportive function by UC-MSCs need to be further investigated since several questions are still open. The first is whether or how cell-to-cell contact and/or secretion of soluble factors influence the entire process and if both mechanisms are needed to achieve maximum efficacy. A comprehensive knowledge of the secreted molecules of WJ-MSCs, through exploiting secretomic approaches, and the design of mechanistic studies aimed to isolate single factors to gain the same effect of using the cells are the next targets in this fascinating field of research. The second aspect which warrants further research is to define the intrinsic functional properties of the expanded UCB-HSPCs, and also to well clarify which sub-populations of HSPCs are generated at different culture conditions in the presence of MSCs. Only with a full knowledge of factors and pathways involved in the process of HSPCs expansion in vitro, optimal protocols can be established to further improve the existing clinical application.

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Compliance with Ethical Standards

Conflict of Interest Giampiero La Rocca is member of the Scientific Board of Auxocell laboratories, Inc. The other authors declare no potential conflicts of interest.

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