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The Therapeutic Potential, Challenges and Future Clinical Directions of Stem Cells from the Wharton's Jelly of the Human Umbilical Cord

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Abstract Mesenchymal stem cells (MSCs) from bone marrow, adult organs and fetuses face the disadvantages of invasive isolation, limited cell numbers and ethical constraints while embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs) face the clinical hurdles of potential immunorejection and tumorigenesis respectively. These challenges have prompted interest in the study and evaluation of stem cells from birth-associated tissues. The umbilical cord (UC) has been the most popular. Hematopoietic stem cells (HSCs) harvested from cord blood have been successfully used for the treatment of hematopoietic diseases. Stem cell populations have also been reported in other compartments of the UC viz., amnion, subamnion, perivascular region, Wharton's jelly, umbilical blood vessel adventia and endothelium. Differences in stemness characteristics between compartments have been reported and hence derivation protocols using whole UC pieces containing all compartments yield mixed stem cell populations with varied characteristics. Stem cells derived directly from the uncontaminated Wharton's jelly (hWJSCs) appear to offer the best clinical utility because of their unique beneficial properties. They are non-controversial, can be harvested painlessly in abundance, proliferative, possess stemness properties that last several passages in vitro, multipotent, hypoimmunogenic and do not induce tumorigenesis even though they have some ESC markers. hWJSCs and its extracts (conditioned medium and lysate) also possess anti-cancer properties and support HSC expansion ex vivo. They are thus attractive autologous or allogeneic agents for the treatment of malignant and non-malignant hematopoietic and non-hematopoietic diseases. This review critically evaluates their therapeutic value, the challenges and future directions for their clinical application.

Keywords Standardization of derivation protocols · Properties and applications of Wharton's jelly stem cells · Umbilical cord compartments

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Introduction

Various types of stem cells have been isolated to date in the human from a variety of tissues including preimplantation embryos, fetuses, birth-associated tissues and adult organs. They can be broadly classified into embryonic stem cells (ESC), mesenchymal stem cells (MSC) and hematopoietic stem cells (HSC) based on biochemical and genomic markers. The plasticity of the stem cells in these three categories are also different, with the most versatile being ESCs which theoretically can be differentiated into almost all tissues in the human body and hence have been labeled as pluripotent or the 'mother of all cells'. Pluripotency in ESCs is defined as the ability of these cells to produce tissues from all three germ layers (ectoderm, mesoderm and endoderm) when transplanted into immunodeficient mice. ESCs are generated from surplus In Vitro Fertilization (IVF) embryos, MSCs from fetal and adult tissues and HSCs from the bone marrow, peripheral and umbilical cord blood (UCB). ESCs, MSCs and HSCs follow established paradigms of human development in vivo governed by the programmed pathways of their genomes.

ESCs, HSCs and MSCs from adult and fetal tissues have their own limitations. ESCs are controversial and their derived tissues pose the risks of immunorejection and tumorigenesis. To overcome the problem of immunorejection protocols were

developed where tissues could be personalized to patients by transfecting the patient's somatic cells with pluripotent genes to produce human induced pluripotent stem cells (hiPSCs) from which desirable tissues could be derived for the patient for transplantation therapy. Viral and non-viral reprogramming methods have been successfully developed but unfortunately epigenetic changes in the form of chromosomal duplications and deletions have been reported in the ensuing hiPSCs [1]. It was postulated that such genetic changes arise at three different points in the reprogramming protocol: (i) those already present in the parent somatic cell that is being reprogrammed; (ii) induced during reprogramming and (iii) induced during serial culture [2]. These genetic changes are probably brought about by in vitro manipulation as the hiPSCs are not given the opportunity to go through the natural established in vivo paradigms of human development. Additionally, hiPSCs induce tumorigenesis in immunodeficient mice and such teratoma formation is faster and more efficient than their hESC counterparts [3]. Hence, until these clinical hurdles are overcome the use of hiPSCs is limited only to the study of the pathogenesis of disease and as platform technology for drug screening and discovery.

Fetal MSCs are controversial as they are derived from human abortuses. MSCs from adult organs have limitations in terms of cell numbers and as such require expansion in vitro running the risk of loss of stemness properties, induction of artifactual chromosomal changes and problems of contamination. HSCs have limited plasticity in that they can differentiate only into blood and blood-related lineages. Also, the HSC numbers harvested from the bone marrow and umbilical cord are low and require ex vivo expansion for the treatment hematopoietic diseases in adult humans.

Stem cells from birth-associated tissues are gaining popularity and have been derived from the placenta, amniotic fluid, amniotic membrane and umbilical cord (UC) [4, 5]. Of these sources, those isolated directly from the Wharton's jelly of the umbilical cord appear to offer greater clinical utility because they are less heterogeneous and possess unique properties over bone marrow MSCs [6]. As such, they will be the focus of this review.

Embryology of the Human Umbilical Cord

Fertilization and the first 4 days of cleavage up to the early blastocyst stage takes place in the Fallopian tubes in the human. On day 5 the early blastocyst descends down into the uterus, continues its divisions, undergoes expansion to the fully expanded blastocyst stage and then implants in the uterine endometrium around day 7 to 9. The migration of cells within the expanded blastocyst results in the laying down of two distinct cell layers, a peripheral layer of trophectoderm (TE) destined to become the placenta and a cluster of approximately 30–50 cells (inner cell mass, ICM) that protrude from the inner wall of the polar TE and destined to form the entire fetus. The ICM later develops into the hypoblast and epiblast. The hypoblast gives rise to the yolk sac and allantois which eventually degenerates and the epiblast cells which are pluripotent give rise to the three germ layers (ectoderm, mesoderm and endoderm) from which the various organs and extra-embryonic membranes (amnion, chorion, placenta and UC) are formed. During further development the amnion forms an outer covering for the UC and the UC carries within it three umbilical blood vessels (two arteries and a vein) to shuttle nutrients between mother and fetus. The amnion comprises of three layers viz., inner epithelial cell layer, an intermediate non-cellular basement membrane and outer mesenchymal layer [5]. The TE forms the cytotrophoblast and syncytiotrophoblast of the placenta while the blastocoelic cavity eventually produces the exocoelom. The part of the UC closest to the fetus may therefore contain remnants of the yolk sac and allantois.

Human epiblast cells express the surface marker antigens SSEA-3 and SSEA-4 [7]. Human embryonic stem cells (hESCs) that originate from the epiblast also express SSEA-3 and -4 but not SSEA-1 [8]. Human epiblast cells also express the pluripotent genomic markers OCT3/4 and NANOG and with continued development as cells differentiate into various lineages, OCT3/4 gets downregulated [7]. Thus, since the UC lies in an intermediate position between embryo and adult on the human developmental map, evaluation of the degree of expression of the members of the entire OCT family in stem cell populations of the UC is urgently needed to understand their role in stem cell plasticity.

The Various Compartments of the Human Umbilical Cord

The human UC starts to develop around the fifth week of gestation and at term has an average length of about 50 cm [9]. From a stem cell derivation point of view, various reports in the literature describe several compartments in the human UC (Fig. 1). Stem cells have been derived in the amniotic compartment (outer epithelial layer and inner subamniotic mesenchymal layer), the Wharton's jelly (WJ) compartment, the perivascular compartment surrounding the blood vessels, the media and adventitia compartment of the walls of umbilical cord blood vessels, the endothelial compartment (inner lining of the vein) and the vascular compartment (blood lying within the umbilical cord blood vessels). All these compartments have been described as distinct regions [10] and the nomenclature used in the literature for these various compartments has been misleading and not standardized, with terms such as 'cord lining', 'subamnion', 'intervascular', 'perivascular' and 'hUVEC' being used. Stem cell populations with varied stemness

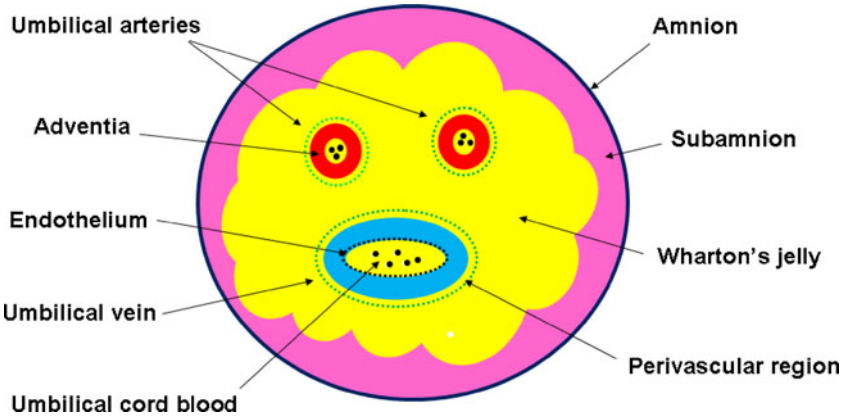

Fig. 1 Diagrammatic illustration of a cross-section of the human umbilical cord showing the compartments from which stem cells have been isolated (amnion, subamnion, Wharton's jelly, perivascular, adventiatia, endothelium and umbilical cord blood)

properties have been reported for each of these compartments [11] but the various individual derivation protocols published in the literature for stem cells from the UC are ambiguous and do not pay heed to the differences in stem cell populations between compartments. At the same time it is not known whether the stem cell populations between compartments are one and the same as there is no clear demarcation histologically between some of these compartments.

Given the reports that stem cell populations in different compartments have varied stemness characteristics the derivation protocols involving entire cord pieces containing all the compartments will result in mixed heterogeneous stem cell populations making a meaningful assessment of investigations difficult. It is therefore urgently necessary to standardize a derivation protocol for MSCs of the UC that yields defined or minimally heterogeneous cell populations.

The Diverse Methods of Derivation of Stem Cells from the Human Umbilical Cord

Several authors have reported the presence of stem cells in the various compartments of the UC using different derivation protocols. At least six different methods have been reported (Fig. 2). (i) UC pieces were first cut open, the umbilical blood vessels (which may carry with them the perivascular regions) removed and the remaining inner surface of the cord piece either scraped or squeezed with forceps to retrieve the WJ from which stem cells were harvested [12, 13] (Fig. 2a). (ii) UC pieces were cut open, umbilical blood vessels retained and only the WJ was separated. The WJ was then either directly exposed to enzymatic solutions to release the cells or cut into small pieces and then enzymatically treated [14-20]. In our laboratory we invert the cord pieces with intact umbilical vessels into an enzymatic solution (collagenase and hyaluronidase) at 37 °C for 30–45 min to facilitate detachment and loosening of the WJ into culture medium and then separate the cells from the WJ by passing the gelatinous masses through a syringe and needle [15, 17] (Fig. 2b). The stem cell populations in (i) and (ii) were referred to as human Wharton's jelly stem cells (hWJSCs) as they were derived directly from the WJ. (iii) Entire UC pieces with intact umbilical blood vessels were cut into smaller pieces and then grown as explants on plastic for a few days after which cell outgrowths from the explants were separated and cultured [21-24] (Fig. 2c). To maximise the recovery of stem cells, Tsagias et al. [25] first washed the entire length of the UC under sterile conditions to remove blood, then sterilized its surface, and with the umbilical blood vessels intact immersed the entire UC into a sterile bag containing an enzymatic solution of collagenase and hyaluronidase and incubated the bag at 37 °C for 3 h with gentle agitation. The UC was then exposed to trypsin for a further 30 min and the digested cell suspension collected by gravity. The stem cell populations in (iii) above were referred to as umbilical cord mesenchymal stem cells (UC-MSCs) (iv) The subamnion region of the UC was removed with a razor blade, cut into small pieces and grown on plastic as explants from which the cell outgrowths were separated and cultured. These stem cell populations were called subamnion or cord lining MSCs [10, 26] (Fig. 2d). These workers claimed that when separating the subamnion region from the rest of the UC it was impossible to completely remove the adjacent region underneath the amniotic epithelium and that dissection with a razor blade would leave a thin layer of mixed tissue. The same group also reported that it was difficult to exclude the contamination of Wharton's jelly-derived cells using their method of dissection. They also reported that their method was extremely time-consuming and required at least two working days after receiving the UCs and stated that it was reasonable to conclude that greater quantities of cells were available from the WJ [10]. (v) The umbilical blood vessels were removed from cord pieces, tied at either end into loops and the loops placed into an enzymatic solution for a specific period of time to allow detachment of cells from the perivascular region which are then grown in culture. These were referred to as UC perivascular stem cells (UCPVSCs) [27] (Fig. 2e).

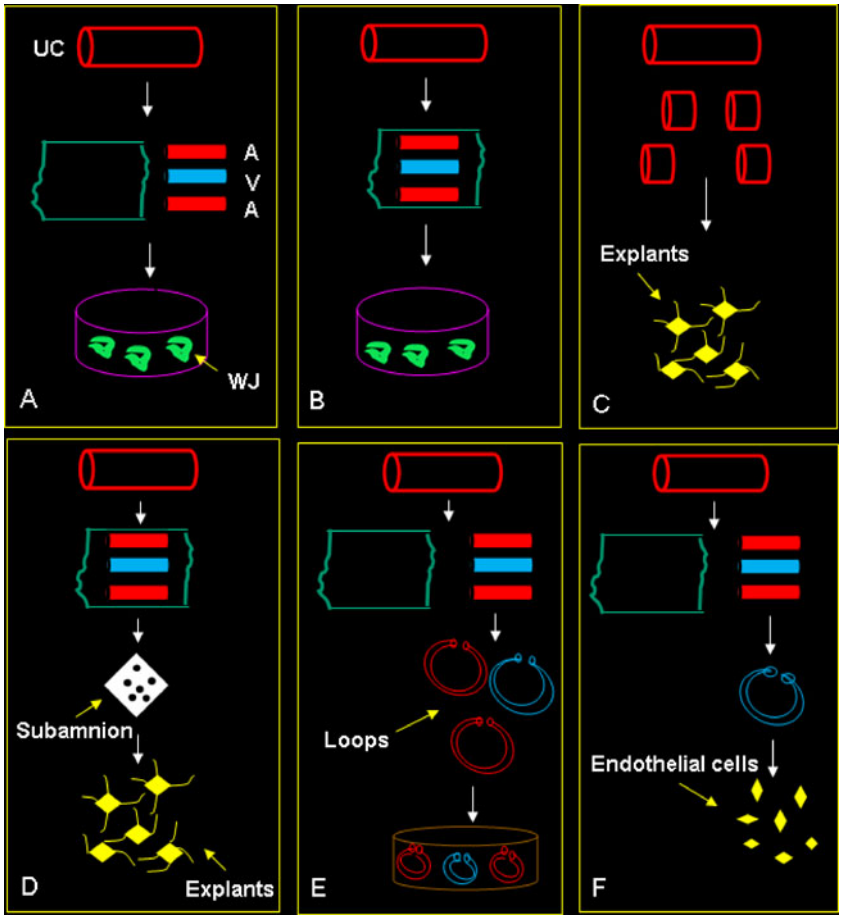

Fig. 2 Diagrammatic illustration showing six different methods of derivation of stem cells from the human umbilical cord published in the literature

(vi) Romanov et al. [28] isolated stem cells from the endothelial lining of the vein of the UC by first removing the vein and then passing through it an enzymatic solution to digest and remove the inner endothelial lining cells. The cell suspension was centrifuged to remove the enzymes and the cell pellet washed and seeded into culture medium in plastic dishes to grow the endothelial cells. The stem cells from such endothelial linings have been commonly referred to as human umbilical vein epithelial cells (hUVECs) (Fig. 2f).

Although debated, it has been claimed that MSCs usually reside in the perivascular regions of organs. In the UC however, it is very difficult to demarcate a perivascular zone from the rest of the intervascular WJ. It would therefore be prudent to keep the umbilical blood vessels intact when enzymatically separating the WJ so that the entire contents of the intervascular compartment (Wharton's jelly and perivascular cells) could be isolated. Removal of the blood vessels may result in loss of perivascular stem cell numbers from the intervascular WJ compartment as they may be firmly attached to the outer wall of the umbilical blood vessels. Watt et al. [29] reported that they observed CD146 + cells in 40 % of the cells in the perivascular regions. Recently, Kikuchi-Taura et al. [20] incubated UC pieces (with intact umbilical blood vessels) in an enzymatic mixture of collagenase and hyaluronidase for 2 h at 37 °C with intermittent shaking and then isolated stem cells from the WJ. They compared their derivation method with the method of Weiss et al. [12] (controls) which was similar except that in the controls the umbilical blood vessels were not removed. They reported large numbers of hWJSCs when the umbilical blood vessels were not removed and claimed that in vitro expansion was not required and that their fresh hWJSCs had the potential to suppress graft versus host disease. We used a similar enzymatic method to that of Kikuchi-Taura et al. [20] but separated the stem cells from the WJ by passing the gelatinous masses through a syringe and needle. We too reported large harvests of hWJSCs of approximately 4.7×10^6 cells per cm of UC [15, 17].

Recently, Bosch et al. [24] diced whole UC pieces into smaller pieces and grew them as explants. The outgrowths from the explants were separated, grown as monolayers and referred to as UC-'MSCs'. These stem cell populations may be heterogeneous and mixed as they originate from many compartments as they were not derived from a specific compartment. Based on this whole cord piece protocol these workers questioned whether UC-derived cells were true MSCs.

Even though MSCs have been reported from the various compartments of the human UC, the compartment with stem cell populations of the most optimal therapeutic value remains debatable. Robust comparisons between the stem cell populations of these various compartments so as to identify the most optimum source and subpopulation is urgently necessary for standardization and comparison of results between groups and to ensure reliability in terms of stemness properties, product quality, safety and efficiency for attaining regulatory approval for future clinical trials. Currently, stem cells from the WJ compartment appear to be the most defined with several unique characteristics.

Stem Cell Populations in the Various Human Umbilical Cord Compartments have Different Stem Cell Characteristics

It is interesting to note that differences in the properties of the stem cell populations in the various UC compartments have been reported. A differential distribution pattern of the various cytoskeletal proteins of stromal cells and extracellular matrix proteins was observed in different zones of the subamniotic stroma, WJ and the adventia of the umbilical blood vessels [30]. Comparisons were made between the MSCs derived from arterial (UCA), venous (UCV) and Wharton's jelly (UCWJ) explants of the human UC. Individual UC components from these three areas were dissected, diced into small fragments and aligned in explant cultures from which migrating cells were separated by trypsinization. UCV cells showed significantly higher frequency of colony-forming units than UCA and UCWJ. When compared for osteogenic potential, UCWJ were the least effective while UCA-derived cells developed alkaline phosphatase activity with or without an osteogenic stimulus [31]. MSCs have also been isolated in small numbers from the UCB compartment which lies within the umbilical blood vessels. However, the existence of such MSC populations in human UCB has been debated. Some workers could not isolate MSCs from UCB [32] while others claimed that UCB and peripheral adult blood were not rich sources of MSCs [33]. Some other reports stated that the typical features of hMSCs in UCB were their low counts per volume of UCB and very low proliferation rates [34-36].

The extra-embryonic membranes harbor a variety of embryonic or premature cell populations such as MSCs, endothelial stem/progenitor cells (EPCs, ECFCs) and HSCs (CD34 + and CD133+) [37]. The same authors emphasized that with respect to MSC populations from the UC, the different parts of the UC should be considered individually. La Rocca et al. [38] reported that there was a plethora of cellular sub-sources and populations that can be derived from the UC and Conconi et al. [11] described in detail the properties of these different cell populations derived by various methods. It has also been emphasized that there are a number of enzyme-mediated and enzyme-free methods described in many publications and patents to derive MSCs from the UC but there was no standardized method that was widely accepted [39, 40]. Additionally, stem cell populations from different UC compartments may represent cell populations that prefer to be differentiated along a specific lineage eg., musculoskeletal tissue engineering [41].

In situ demonstration of MSC markers on cryosections of whole UC pieces was reported by Schugar et al. [22]. After histological and immunohistochemical (IHC) analysis using primary and secondary antibodies for the various CD markers, these workers showed that the vascular regions could be clearly distinguished from the WJ matrix and epithelium. CD34+ and CD144+ cells were detected in the endothelial lining of the umbilical vessels but not in other regions of the UC. CD146 expression was observed in the vessel walls and perivascular regions. CD44+ and CD105+ cells were detected in the vessels, perivascular regions and outer WJ matrix and CD73+ cells were highest in the epithelium and subepithelial regions. These results confirm differences in stemness properties in cells of the various UC compartments. The differences are further confounded by the differences in derivation methods. For example, mechanical disruption, explant culture and dispase digestion yielded CD144+/CD146+ endothelial cells in abundance [42, 43].

Some workers claimed that of the MSCs isolated from the cord blood, WJ and perivascular regions of the UC, those from the WJ (which they included as intervascular and subamnion) offered better clinical utility because isolation frequency of colony forming unit-fibroblasts (CFU-Fs) were extremely high and delays in processing did not impact isolation [44]. The same authors stated that the MSCs from the perivascular region despite having high proliferative potential had limited transdifferentiation potential. Marker and differentiation assays indicated that the mesenchyme became more differentiated and mature from the subamnion to the perivascular regions [44, 45].

Origin of hWJSCs: How Did They Arrive in the Wharton's Jelly and What Are They Doing There?

Two possible theories can be presented as to how stem cells arrived in the WJ and what their possible role is during gestation. Wang et al. [46] carried out some elegant studies on whole human conceptuses (40 days old) that were collected through RU984-induced termination of pregnancies (TOPs). They dissected out the cells from the fetal bone marrow, yolk sac and aorta-gonadal mesonephros (AGM) and characterized them for the conventional MSC markers

recommended by the International Society for Cellular Therapy [47]. The cells in all three regions were positive for the MSC markers and based on their findings they postulated that there were two waves of migration of fetal MSCs in early human development. In the first wave, MSCs migrated from the yolk-sac and AGM via the UC to the placenta and in a second migration MSCs reverse-migrated from the placenta via the UC to home in the fetal liver and bone marrow. During these waves of migration it was suggested that some of these MSCs got trapped and resided in the gelatinous WJ of the UC [46, 48]. Their stemness characteristics appear to get modified while in their new environment making them different from bone marrow MSCs (hBMMSCs).

A second hypothesis is that the cells in the WJ are actually primitive mesenchymal stromal cells (myofibroblasts) originating from mesenchyme that was already there within the UC matrix. The role of these cells was probably to secrete the various glycoproteins, mucopolysaccharides, glycosaminoglycans (GAGs) and extracellular matrix (ECM) proteins to form a gelatinous ground substance to prevent strangulation of the umbilical blood vessels during gestation. Being primitive stromal cells and epiblast in origin they probably acquired both ESC and MSC markers at different levels of expression. Based on embryological origin and migration, the gelatinous WJ may be different from other stem cells in the UC compartments in terms of their secretory profiles. Meyer et al. [49] reported that the WJ was rich in mucoplyscacchrides and possessed a network of glycoprotein and collagen microfibrils. Several bioactive molecules (interferons, growth factors, interleukins, GAGs, cell adhesion molecules) present in the secretions released by hWJSCs [18, 50, 51] appear to be the building blocks for immunomodulatory mechanisms and tissue repair and thus can be taken advantage of for the repair of bone, cartilage and joint defects.

Wharton's Jelly Stem Cells have a Common Origin to Bone Marrow MSCs But are Different

hBMMSCs are the most popular and widely used MSCs for human stem cell research and clinical application. However, the harvesting of bone marrow MSCs is invasive and painful with the risk of infection and donor site morbidity. These features have resulted in a decline of allogeneic donations of hBMMSCs and the search for alternative MSC sources that are non-invasive.

Interestingly, even though hWJSCs and hBMMSCs may have common origins during human embryonic and fetal development, hWJSCs appear to be distinctly different and have advantages over hBMMSCs when it comes to clinical application [52]. hWJSCs resemble hBMMSCs in terms of a short-fibroblast-like phenotype [15, 17, 43, 53], non-hematopoietic surface markers [53], hypoimmunogenecity [54, 55], multipotent plasticity [38, 56-58] and expression of some markers such as CD90, CD105, CD13, CD73, CD10, CD29 CD51, CD166, CD44 and the HLS antigens HLA-A, B, C and G.

However, unlike hBMMSCs, hWJSCs do not express CD45, CD14, CD56, CD31 and CD34 at high levels and are HLA DR+[29], have higher proliferation rates, increased colony forming unit (CFU) formation and stemness characteristics that last for longer periods of time after serial passaging [17, 59]. CD68 which is classically recognised as a macrophage marker was shown to be highly expressed in hWJSCs [60]. Additionally, unlike hBMMSCs, hWJSCs express several ESC markers at different levels of expression such as the members of the OCT family, embryonic surface marker antigens (SSEA-4, Tra-1-60 and Tra-1-81), alkaline phosphatase (ALP), DNMT3B and GABRB3 and the genomic markers (SOX2, NANOG, REX2) [11, 61-64]. Based on a review of published literature, Conconi et al. [11] tabulated a detailed comparison of the different biochemical markers identified for stem cells derived by various methods from the different UC compartments [subendothelium (SE), perivascular (PV), umbilical cord lining (UCL), Wharton's jelly (WJ) and whole umbilical cord (wUC)]. They concluded that the stem cell populations from the various compartments had two common features in that they did not possess a hematopoietic profile and HLA class II antigens, but MSC and ESC markers were expressed differently in stem cell populations between compartments. CD133 and CD235a were expressed in wUC but not in the other compartments. Stem cells from UCL and partially from the PV compartments expressed CD14 which was not seen in stem cells from WJ, SE and wUC. Differences were also noted for OCT4, SSEA-4, STRO-1 and integrin-α for the stem cell populations in these different compartments. The authors claimed that the findings suggest that each part of the human UC may contain a MSC population that differs from those of other parts. In general, most primitive somatic cells possess greater growth rates in vitro than mature cells. This primitive cell characteristic appears to apply to MSCs isolated from the UC as they are highly proliferative.

hWJSCs have MSC and ESC Markers

hWJSCs share some of the stemness characteristics of both ESCs and adult MSCs as described above by possessing high level expression of MSC-CD markers and varied levels of expression of different ESC markers. They probably inherit some of the ESC markers because the UC lies in between the embryo and adult organs on the developmental map [5]. It would therefore be reasonable to refer to them as

'intermediate stem cells' that have retained some ESC markers while evolving into fully-fledged MSCs that satisfy all the minimum criteria for MSCs recommended by the International Society of Cell Therapy such as plastic adherence, self-renewal, CD-marker expression and ability to differentiate into adipocytes, chondrocytes and osteocytes [47]. Since hWJSCs originate from the epiblast theoretically they would be expected to be pluripotent. Cells that possess pluripotency usually result in chaotic differentiation of all three primordial germ layers to produce teratomas in vivo. However, hWJSCs do not produce teratomas in immunodeficient mice or immunocompetent non-human primates when transplanted at high cell doses with/without matrigel (matrigel is known to encourage teratoma formation in vivo) via various administrative routes [65, 66]. Hence, hWJSCs may have lost the property of pluripotency or their upregulated tumour suppressor genes [61] are able to downregulate their pluripotent genes, or the expression levels of their pluripotent genes are inadequate to induce tumorigenesis. Interestingly, non-tumorigenic hESCs were generated when hESCs were grown on hWJSC feeders [19] suggesting perhaps that unique proteins released by the tumour suppressor genes in hWJSCs are able to control the tumorigenic potential of hESCs. These interesting hypotheses warrant further investigation.

Cell Numbers and Growth Characteristics of hWJSCs

It is well known that with manipulation of cells in vitro there are risks of their genotype being altered. With continuous passaging cells undergo major and minor chromosomal changes and these changes occur at different passage numbers between different cell types (see human cell lines in ATCC collection, Maryland, USA). Human fallopian tubal ampullary cells started to produce polyploid chromosome complements after the 20th passage [67]. hESCs and hiPSCs were shown to develop deletions and duplications with early serial passaging [1] and Ben-David and Benvinisty [2] postulated that such chromosomal changes could be induced at several stages during the reprogramming protocol of somatic cells to hiPSCs in vitro. Thus, the genomic stability of passaged UC-MSCs must be assessed to ensure that the most stable passage, free of genomic anomalies is used for clinical application. Such changes in genotype may arise at different passage numbers between the various stem cell populations in the various UC compartments.

Thus, minimal manipulation of stem cells during ex vivo expansion would be preferred to avoid such problems. To date, our group have successfully derived 22 hWJSC lines from 22 human umbilical cords (100 %) and could consistently recover approximately 5×10^6 fresh live hWJSCs/cm of umbilical cord directly from the WJ before culture. Hence from a full-term UC of 50 cm a total of 2.5×10^8 hWJSCs could be theoretically harvested without culture. The use of a complex culture medium comprising of DMEM-high glucose+Knockout (KO) serum supplemented with bovine fibroblast growth factor (bFGF), insulin-transferrin-selenium (ITS) and L-glutamine generated high proliferation rates with mean population doubling times (PDT) of around 24 h. The stemness markers of the hWJSCs in this culture environment were retained with no chromosomal changes for at least 20 passages. hWJSCs are adherent cells that attach very well to plastic surfaces both in primary culture and passages and thus do not require feeder cells or cell attachment matrices like matrigel for their derivation and propagation. Other workers have also reported 250–300 fold increases in hWJSC numbers that were reached within 6–7 passages with a stable karyotype [45]. These workers reported that a total of $3.6\times10^6\pm6\times10^5$ viable hWJSCs/cm per sample could be obtained after primary culture and the total number of cells expanded to $11.5\times10^8\pm2.3\times10^6$ cells after subsequent passages.

The number of fresh live hWJSCs that could be harvested per cm of umbilical cord seems to vary with different reports because of differences in the derivation methods used. Wang et al. (2004) [14] obtained 25×10^3 cells/cm of human umbilical cord while Weiss et al. [12] obtained 1.5×10^6 cells/cm and Fu et al. [68] obtained 50×10^3 cells/cm of human umbilical cord. The derivation efficiency for hWJSCs from WJ is very much higher (100 %) than the derivation rates of MSCs from other compartments of the UC such as the endothelial and subendothelial layers of the umbilical veins (30 %) [28]. Also, the PDT of human umbilical cord perivascular stem cells (hUCPVSCs) was longer in a simple unsupplemented medium (60 h) resulting in the production of 10^{10} cells within 30 days of culture [27]. Lu et al. [21] showed that the mean PDT of hWJSCs grown in a super-complex medium containing DMEM (low glucose), 5 % FBS, glutamine, antibiotic-antimycotic mixture, VEGF and EGF (instead of bFGF and ITS) was 24 h and remained approximately constant until passage 10 (P10) after which it increased until passage 30 (P30).

Changes in cell surface markers of hWJSCs were observed after in vitro expansion [20]. These workers showed that CD44, CD105 and CD73 expression was increased at passage 4 (P4) and in contrast CD29 and CD90 expression did not change. Garzon et al. [69] showed that the highest viability levels for hWJSCs corresponded to the 5th and 6th passages. They also reported through gene expression analysis that this cell viability was significantly associated with pro- and anti-apoptotic genes. They suggested that there was a complex live-death equilibrium in hWJSCs maintained in culture for multiple cell passages. They concluded that the most optimal passage of hWJSCs that would be of therapeutic value was between the 5th and 6th passage.

Changes in stemness properties and protein secretions during serial culture of hWJSCs have been reported [18].

These workers characterized the proteomes of four passages (P2, P4, P8 and P12) and identified 158 unique proteins. These proteins were classified into 5 functional categories (cytoskeleton and motility, metabolism, protein biosynthesis folding and degradation, nucleotide biosynthesis, and cell signaling). They also observed that certain proteins (shootin 1, adenylate kinase 5 isoenzyme and plasminogen activator-inhibitor 2) were no longer expressed after the 2nd passage. At the end of their culture period (P12) they observed the synthesis of new proteins such as ERO1-like protein alpha, aspartyl-tRNA synthetase and prolyl-4-hydroxylase. Such protein changes between passages highlights the importance of evaluating the most optimum passage for stemness and utility of hWJSCs when it comes to clinical application.

It is also important to standardize the constituents of the culture medium that is used to derive and propagate hWJSCs as the culture environment appears to influence the nature and properties of the cells. Growth factors in the culture medium may have a positive influence on the proliferation rates of these cells in vitro. Culture media that have been used to propagate UC-MSCs have ranged from simple unsupplemented basal media to supplemented super-complex media containing a multitude of supplements. It is not known what additional benefits or detrimental effects exist when there is over-supplementation. The simple media conventionally contain the salts (DMEM), energy substrates (high or low glucose), proteins (fetal bovine serum), L-glutamine and antibiotic-antimycotic mixture [27]. The complex media are similar to the simple media but are supplemented with FGF and ITS [58]. The super-complex media contain additional nutrients such as epidermal growth factor (EGF), platelet-derived growth factor (PDGF), ascorbic acid and dexamethasone [20]. Ascorbic acid and dexamethasone are traditionally used as differentiation agents to drive stem cells towards osteogenic or chondrogenic lineages and as such it is not known whether the use of such agents will compromise the differentiation of hWJSCs into other desirable lineages. Most of the culture media that is used to grow UC-MSCs have ingredients of animal origin (xenoproteins) such as bovine brain extracts, fetal bovine serum and animal sources of insulin and FGF. If hWJSCs are to be taken to the clinic it would be mandatory to develop safe clinical grade current good manufacturing practice (cGMP) cell lines by avoiding the use of these xenoproteins and placing emphasis on human-based ingredients or supplements derived from recombinant DNA technology.

Plasticity of hWJSCs

Wharton's jelly stem cells (WJSCs) can be differentiated into many desirable tissues. When WJSCs were grown in rat neuronal conditioned medium they differentiated into CD11b microglial cells, generated neuronal proteins and the astrocyte protein GFAP was upregulated [68]. When WJSCs were exposed to salvia miltorrhiza (a shade growing perennial flowering plant) they expressed the neuronal markers β-tubulin 111, neurofilament and GFAP [70]. Several groups have reported protocols to differentiate hWJSCs into neurons [14, 15, 71]. In most of these protocols the hWJSCs were first incubated overnight with bFGF and then exposed to dimethyl sulfoxide (DMSO) and butylated hydroxyanisole (BHA). After 5–6 h the hWJSCs were treated with a mixture of potassium chloride, valproic acid, forskolin, hydrocortisone and insulin for long term induction and maintenance of neuronal differentiation [15, 17].

Several groups have also differentiated hWJSCs into bone, cartilage, and adipose cells [14, 27, 45, 72]. When hWJSCs were treated with 5-azacytidine for 3 weeks they started to exhibit the cardiomyocyte phenotype and expressed cardiac troponin 1, connexin 43, and desmin which are the conventional markers for cardiomyocyte differentiation [14]. When hWJSCs were first treated in vitro with vascular endothelial growth factor (VEGF) and bFGF and then transplanted into mouse ischaemic hearts they were shown to differentiate into endothelial cells [73]. hWJSCs exposed to a myogenic culture medium expressed the Myf-5 marker for skeletal muscle on day 7, Myo-D on day 11 and produced long multinucleated cells. When these multinucleated cells were injected into damaged rat muscles they produced the sarcomeric tropomyosin protein [72]. Pancreatic islet-like clusters have also been derived from hWJSCs for the control of diabetes [74, 75].

Immunogenecity of hWJSCs

Stem cells harvested directly from the Wharton's jelly compartment of the human UC have been shown to possess hypoimmunogenic properties that have been characterized both in vitro and in vivo. Weiss et al. [54] showed that hWJSCs inhibited a splenocyte response to concanavalin A stimulation in vitro, do not stimulate T-cell proliferation in a one-way mixed lymphocyte reaction (MLR) assay and inhibit the proliferation of stimulated T cells in a two way MLR assay. They further showed that hWJSCs express mRNA for pan-HLA-G and do not express the co-stimulatory surface antigens CD40, CD80, and CD86. These workers concluded that their results supported the view that there was no evidence of frank immunorejection of undifferentiated hWJSCs and that they would be tolerated in allogeneic transplantation settings.

When pig UC-MSCs were injected into the brains of Parkinsonian rats they were not rejected but survived in the rat brains and multiplied up to 4 weeks and produced

tyrosine hydroxylase positive neurons that expressed porcine-specific markers [76]. When hWJSCs were injected into immunodeficient rat spinal cords, the hWJSCs survived for 16 weeks post-transplantation [77]. In a more recent study, our group showed that when hWJSCs were injected into immunodeficient mice via various routes of administration (intramuscular, subcutaneous, intraperitoneal) they survived in the animals up to the termination of the experiment (20 weeks). The presence of the hWJSCs at the sites of injection were confirmed at 20 weeks by the presence of the human nuclear antigen (HNA). Analysis of the blood and spleen of the animals showed increased levels of anti-inflammatory cytokines [65].

The hypoimmunogenecity of MSCs from other compartments of the human UC derived by different methods have also been reported. The immunogenecity and immunomodulatory properties of umbilical cord lining (subamnion) MSCs were studied by Deuse et al. [78]. When these workers compared the immunogenecity of hBMMSCs and their subamniotic MSCs in immunocompetent mice the hBMMSCs exhibited a faster immunorejection response whereas in immunodeficient mice cell survival was prolonged and similar for both hBMMSCs and subamniotic MSCs. In another derivation method where diced explants of whole UC pieces were grown on plastic and the ensuing cell outgrowths separated and grown as UC-MSCs, HLA-DR and the costimulatory molecules CD80 and CD86 were not expressed by the UC-MSCs. These UC-MSCs also constitutively expressed B7-H1 (a negative regulator of T-cell activation) and its expression increased after interferon-γ treatment in vitro [55]. The immunological studies of stem cells derived from the various compartments of the human UC by different methods have been reviewed comprehensively by Conconi et al. [11] and Prasanna and Jahnavi [44].

Differences between hBMMSCs and hWJSCs in terms of immunogenecity have also been demonstrated. Mild lymphoproliferative responses to BMMSCs but none to hWJSCs were reported when these two cell types were cocultured with human peripheral blood mononuclear cells [79].

Non-tumorigenicity of hWJSCs

Using detailed microarray transcriptome profiling our group reported that hWJSCs possessed different levels of expression of pluripotent ESC genes and high level expression of a family of tumour suppressor and immunogenic genes that perhaps confer on them non-tumorigenic and hypoimmunogenic properties [61]. Other groups reported modest to high level expression of ESC markers in stem cells isolated from the subamnion, amnion and umbilical cord matrix [26, 53, 80, 81]. Given these different levels of ESC gene expression it is important that comparative studies be undertaken to evaluate the tumorigenic potential of stem cell populations from each compartment of the UC.

We reported that when hWJSCs at high doses (5×10^6 cells per injection) were transplanted with matrigel into SCID mice via three different routes and monitored for 20 weeks none of the animals developed tumors [65]. The absence of teratoma formation in the mice suggests that either the expression levels of the pluripotent genes were unable to induce teratomas or that the upregulated tumor suppressor genes are able to over-ride the expression of the pluripotent genes .

Recently, Wang et al. [66] confirmed the safety of human UC-MSCs (hUC-MSCs) in non-human primates. hUC-MSCs at doses of 2×10^6 and 1×10^7 cells/kg were injected intravenously once every two weeks for 6 weeks in cynomolgus monkeys. The toxicity of the cells were evaluated using a battery of parameters (clinical observations, histopathology, blood counts, clinical biochemistry, urine analysis, bone marrow smears and immunology). No stem cell transplantation-related toxicity was reported and all the injection sites and organs studied were normal with no prevalence of tumours.

Furthermore, injection of UC-MSCs or their derived tissues into xenograft diseased rat models resulted in engraftment and good functional outcome with no immunorejection or tumorigenesis [56].

Clinical Applications of hWJSCs and Its Extracts (Conditioned Medium and Cell-free Lysate)

Cell-based Therapies

hWJSCs have specific use for cell based therapies, as an anticancer agent and as stromal support for the expansion of CD34+ cells in cord blood banks. Fan et al. [56] reviewed several studies that undertook the preclinical validation of UC-MSCs or its derived tissues in diseased animal models. In all these studies the UC-MSCs differentiated and engrafted with successful functional outcome in vivo in rat models for cerebral ischemia, intracerebral hemorrhage, spinal cord injury, Parkinson's disease, retinal disease, Type 1 diabetes and myogenic disease.

Anticancer Effects

Many groups have reported that hWJSCs, its conditioned medium (hWJSC-CM) and cell-free lysate (hWJSC-CL) exhibit anticancer effects on solid tumors and are therefore attractive candidates for future cancer therapies [51, 82-88]. When hWJSCs were injected intravenously into severely combined immunodeficient (SCID) mice with mammary adenocarcinomas they migrated to metastatic tumor sites in

the lungs suggesting their homing abilities [80]. When rat hWJSCs were injected intravenously or intra-tumorally into rats with mammary carcinomas they completely abrogated cancer cell growth in 34–38 days compared to controls [84]. Similar findings were later observed in the human. When hWJSCs were administered intravenously 8 days after tumor transplantation in a human mammary adenocarcinoma xenograft rat model they homed to metastatic tumor sites in the lungs and reduced tumor burden [83, 85]. Engineered hWJSCs expressing human interferon-β were also shown to abrogate mammary adenocarcinoma growth in animal models [89]. The anticancer effects of hWJSCs are mediated via cell-to-cell and/or non-cellular contact mechanisms. One of the mechanisms involved in the inhibition of mammary adenocarcinoma cells by hWJSCs in vitro was through entosis [85]. These authors showed with time lapse imaging hWJSCs being first engulfed by mammary adenocarcinoma cells and then disintegrating within the cancer cells leading to apoptosis of the mammary adenocarcinoma cells [87]. The extracellular matrix of WJSCs was also shown to inhibit mammary adenocarcinoma cell proliferation by secretion of dickkopf-1 and suppression of the Wnt signalling pathway [86].

Artifactual growth advantages of one cell type over the other in vitro make coculture (the interaction of two cell types) studies sometimes unreliable. As such there has been recent interest in the evaluation of acellular agents harvested from hWJSCs (hWJSC-CM and hWJSC-CL) for the inhibition of cancer cell growth. Such acellular agents would be less controversial to regulatory bodies and more attractive as patentable technology for clinical application due to the lack of cell therapy induced cancer risks.

Our group examined the inhibitory effects of hWJSC-CM and hWJSC-CL on three cancer cell lines [breast adenocarcinoma (MDA-MB-231), ovarian carcinoma (TOV-112D) and osteosarcoma (MG-63)] [51]. The cancer cells were exposed to either 50 % hWJSC-CM or hWJSC-CL (15 μg/ml protein) for 48–72 h and morphologic changes, cell proliferation, cell cycle, gene expression, cell migration and cell death evaluated. The anticancer effect was most severe on the MG-63 osteosarcoma cell line. The growth of all three cancer cell lines was inhibited with morphologic changes ranging from cell shrinkage to blebbing and vacuolation. Based on MTT and BrDU assays, inhibition of cancer cell growth ranged from 2–6 % and 30–60 % for hWJSC-CM and hWJSC–CL respectively. The transwell migration assay also showed inhibition rates of 20 %–26 % and 31 %–46 % for hWJSC-CM and hWJSC-CL respectively for all three cancer cell lines. The sub-G1 and G2/M phases were increased in cell cycle assays and Annexin V-FITC and TUNEL positive cells were seen in the ovarian and breast cancer cells suggestive of apoptosis. The presence of anti-BECLIN1 and anti-LC3B antibodies seen with the osteosarcoma cells suggested an autophagic mechanism of cell death. There was an upregulation of the pro-apoptotic BAX gene and downregulation of the anti-apoptotic BCL2 and SURVIVIN genes in all three cancer cell lines and the autophagy genes (ATG5, ATG7, BECLIN1) were upregulated in the osteosarcoma cells. We concluded that hWJSC-CM and hWJSC-CL possessed tumour inhibitory properties and may be useful therapeutic anticancer agents [51].

Various molecules in conditioned media and cell-free lysates influence the initiation of transcriptional activity, differential expression of functional genes and reprogramming of specific cell types [90, 91]. However, the exact mechanisms as to how these molecules alter cell fate are not well known. Our data showed that the cell-free lysate had greater tumour inhibitory activity than the cell conditioned medium. This could be attributed to regulatory molecules present within the hWJSC matrix rather than the molecules secreted by hWJSCs into the culture medium because of varying sizes and cellular trafficking.

hWJSCs secrete a wide variety of bioactive soluble molecules including cytokines, glycosoaminoglycans (GAGs), hyaluronic acid (HA), chondroitin sulphate, cell adhesion molecules and growth factors [18, 51, 92, 93]. Cytokines are known to influence cell cycle regulation of cancer cells and induce growth attenuation and apoptosis. Matsuzuka et al. [94] demonstrated that hWJSCs expressing the interferon-β gene significantly attenuated bronchioalveolar carcinoma xenografts in SCID mice. In our xenotransplantation studies we showed increased expression of IL-16 in hWJSCs [65] and high concentrations of interleukins (IL-1a, IL-6, IL-7, IL-8) in hWJSC-CM [51]. We postulated that hWJSC-CM and hWJSC-CL exert their influence on cell cycle regulation leading to cell cycle arrest through cytokine-mediated mechanisms.

It was also reported in a xenograft model that human mammary carcinomas produced in mice with cancer stem cells (CSCs) isolated from the MDA-MB-231 breast cancer cell line underwent regression when injected intra-tumorally with hWJSCs. It was postulated that the inhibitory effects on the human CSC-induced tumours were via inhibition of phosphoinositide 3-kinase and AKT signaling mechanisms [88]. We also observed that hWJSCs do not transform into a tumour-associated fibroblast (TAF) phenotype when exposed to breast and ovarian cancer cells unlike hBMMSCs and as such do not participate in tumour formation [95].

The above data showed that hWJSCs and its extracts have paradoxical anticancer properties. This is quite an exceptional feature because the claims of anticancer properties of MSCs in general have been controversial. Furthermore, small animal xenograft models to study complete abrogration of tumours usually provide inconclusive results as most animal care guidelines do not encourage the maintenance and monitoring of animals with tumours for long periods of time because of undue distress to the animals. As such, larger xenograft animal models preferably non-human

primates will provide more conclusive evidence that hWJSCs and/or its extracts abolish tumours completely. Although the above studies showed that hWJSCs and/or its extracts were able to shrink tumours in both size and volume, complete abolishment of the tumours need to be demonstrated with doses that match the human if this novel approach to cancer therapy is to be adopted in clinical settings. Also, the actual molecules responsible for the anticancer effects of hWJSCs need to be closely examined through rigorous exosomal microRNA and proteomics studies.

hWJSCs and Its Extracts Support for Ex Vivo Expansion of HSCs

Confluent cell monolayers from three primary cell types (hBMMSCs, hUVEC and hWJSCs) were compared as feeder stromal cell support for the expansion of hematopoietic cells isolated from UCB [96]. Mononuclear cell expansion was 30–60 fold, colony-forming cell expansion 20-40-fold, and cobblestone area-forming cell expansion was 10-50-fold in the presence of these monolayers. After evaluating the immunological properties of these three primary feeder cell types the authors concluded that all three cell types may be suitable for use in clinical settings for UCB-CD34+ expansion with hWJSCs being the preferred choice because of easier and more efficient methods of isolation. The authors added that the choice of feeder stromal support should rest on whether an autologous coculture is wanted or not.

Our group showed through time lapse imaging that in the presence of hWJSCs and hWJSC-CM, HSCs put out pseudopodia-like outgrowths, became highly motile, migrated towards and attached to the surfaces of hWJSC monolayers and underwent proliferation [50]. After 9 days of culture, MTT and trypan blue proliferation and viability assays showed significant increases in HSC numbers. FACS analysis showed significantly greater numbers of $CD34^+$ cells compared to controls. We also reported that in the presence of hWJSC-CM, HSCs produced the highest number of colonies (CFU assay) and all the six typical classifications of colony morphology suggestive of normal hematopoiesis were observed [50]. Proteomic analysis of the hWJSC-CM showed significantly greater levels of interleukins (IL-1a, IL-6, IL-7 IL-8), SCF, HGF and ICAM-1 compared to controls suggesting that they may be the agents involved in the HSC and CD34+ expansion [51].

The above preliminary studies demonstrate the exceptional properties of hWJSCs over other MSC types. More in-depth controlled studies are required showing significant fold increases in HSC numbers that would be of therapeutic value before the results are definite and can be applied in clinical settings. If this is definitely known then we propose that cord blood banks freeze from the same umbilical cord autologous hWJSCs at the same time when cord blood HSCs are being frozen for future ex vivo HSC expansion and cell based therapies of the patient when required (Fig. 3).

Challenges and Future Directions

Based on the results of in vitro laboratory studies and preclinical animal validation already carried out on hWJSCs by

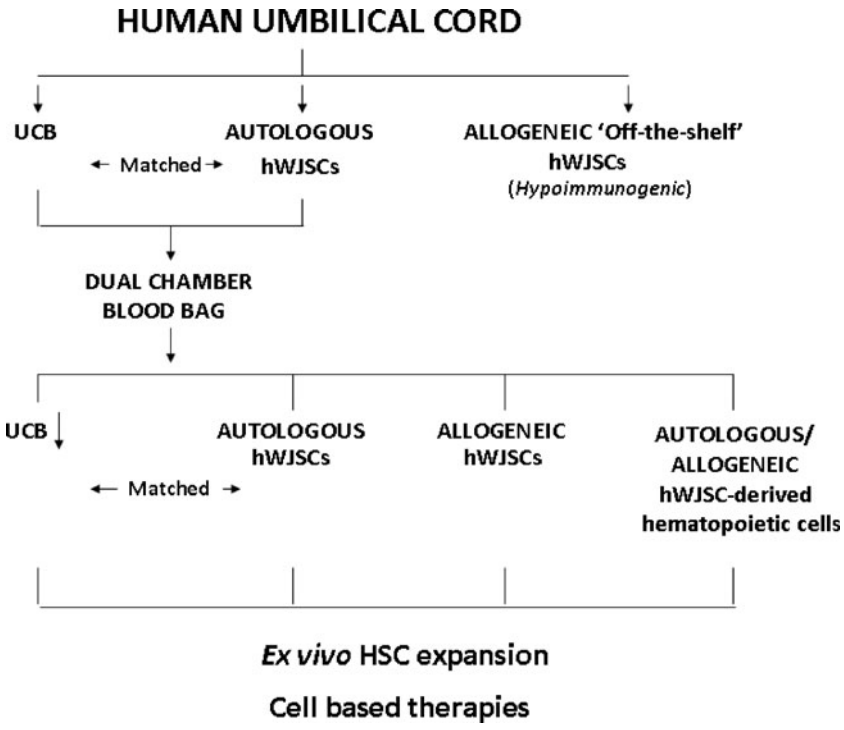

Fig. 3 Proposed model for storage and use of human Wharton's jelly stem cells in cord blood banks for autologous and allogeneic cell based therapies

various groups it appears that hWJSCs and/or its extracts (hWJSC-CM and hWJSC-CL) may be ideal agents for the treatment of malignant and non-malignant diseases beyond the hematopoietic system. The major challenge is to translate and confirm whether the same results obtained pre-clinically will be observed in human clinical settings. To meet this objective the next step would be the preparation and storage of clinical-grade hWJSCs and its extracts in current good manufacturing practice (cGMP) conditions to ensure that they are safe for clinical application. Thereafter, such cGMP-compliant hWJSCs/extracts could be used first in Phase 0 clinical trials to confirm patient safety and improved functional outcome before proceeding to Phase II and III trials. Given the fact that work has already shown that hWJSCs are safe and non-tumorigenic in both laboratory animals and non-human primates it may not be difficult to obtain approval from regulatory bodies to administer fresh or early passaged cGMP-compliant hWJSCs to patients in Phase 0 trials. As a first step to taking these agents to the clinic it may be quicker to obtain regulatory approval for hWJSC extracts rather than hWJSCs because of lesser concerns of any cell-associated cancer risks.

The routes of administration, cell numbers, volumes and duration of dosages are some of the factors that remain challenges and need to be worked out to bring about effective treatment. It is also not known whether for cell based therapies hWJSCs need to be differentiated first in vitro into the desirable tissue of choice before transplantation or whether they can be transplanted directly into the patient allowing differentiation and engraftment to take place in vivo. It is also not known whether any improvements in functional outcome will be mediated via engraftment of differentiated tissues or via paracrine effects as is the case with autologous bone marrow MSC transplantation. Another major challenge is to evaluate in clinical trials whether allogeneic hWJSCs will engraft successfully in the human as they have been shown to have hypoimmunogenic properties.

Although it has been shown in laboratory animal xenograft models that certain tumors shrink in size after exposure to hWJSCs/extracts it has not been possible in most studies to monitor such animals for long periods of time to evaluate complete tumor abolishment because of possible distress caused by the tumors to the animals. Therefore, to confirm complete abolishment of tumors with hWJSCs/extracts it may be important to evaluate dose-time studies on larger non-human primate models where monitoring can be done for longer periods of time until the tumors are completely abrogated. However, the published results of many studies showing the positive anticancer effects of hWJSCs/extracts may be adequate justification to make a case for human clinical application at least in Phase 0 trials. The tumors that have been studied thus far could be first shrunk with the administration of cGMP grade hWJSCs/extracts and then followed with chemotherapy or surgery. It is not known whether hWJSCs/extracts specifically target cancer stem cells (CSCs) residing within tumors. Future anti-cancer studies should be aimed in this direction which if positive may change the paradigms of cancer therapy where hWJSCs/extracts are used to target CSCs that usually reside at the core of tumors rather than at peripheral tumorigenic cells that are the actual progeny of the CSCs.

The derivation and storage of autologous hWJSCs/extracts on the same day that UCB-HSCs are frozen from the same UC will serve as an ideal adjuvant in cord blood banks for future personalized cell based therapies and expansion of HSCs for the patient and immediate family. Autologous hWJSCs/extracts from the same umbilical cord have the advantage of being matched to the same patient avoiding any immunorejection problems. Also, since hWJSCs have been shown to be hypoimmunogenic, allogeneic sources of hWJSCs/extracts from donor umbilical cords would also serve as useful 'off-the-shelf' cells for the same purposes. It is convenient to freeze and store the hWJSCs/extracts in the second chamber of a dual chamber blood bag with the first chamber reserved for the same patient's UCB. Since hWJSCs are highly proliferative the frozen hWJSCs could be thawed and confluent monolayers established within a few hours as stromal support for expansion of HSCs or be used for cell based therapies. hWJSC-CM or hWJSC-CL being cell-free liquids could also be separately frozen in ampoules or cryovials in the liquid nitrogen vapor phase and after thawing be used immediately. Currently, most cord blood banks discard and do not freeze UCB samples that have low HSC counts. Storage of the hWJSCs/extracts provides an opportunity to salvage such samples as they can be expanded with the hWJSCs/extracts.

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