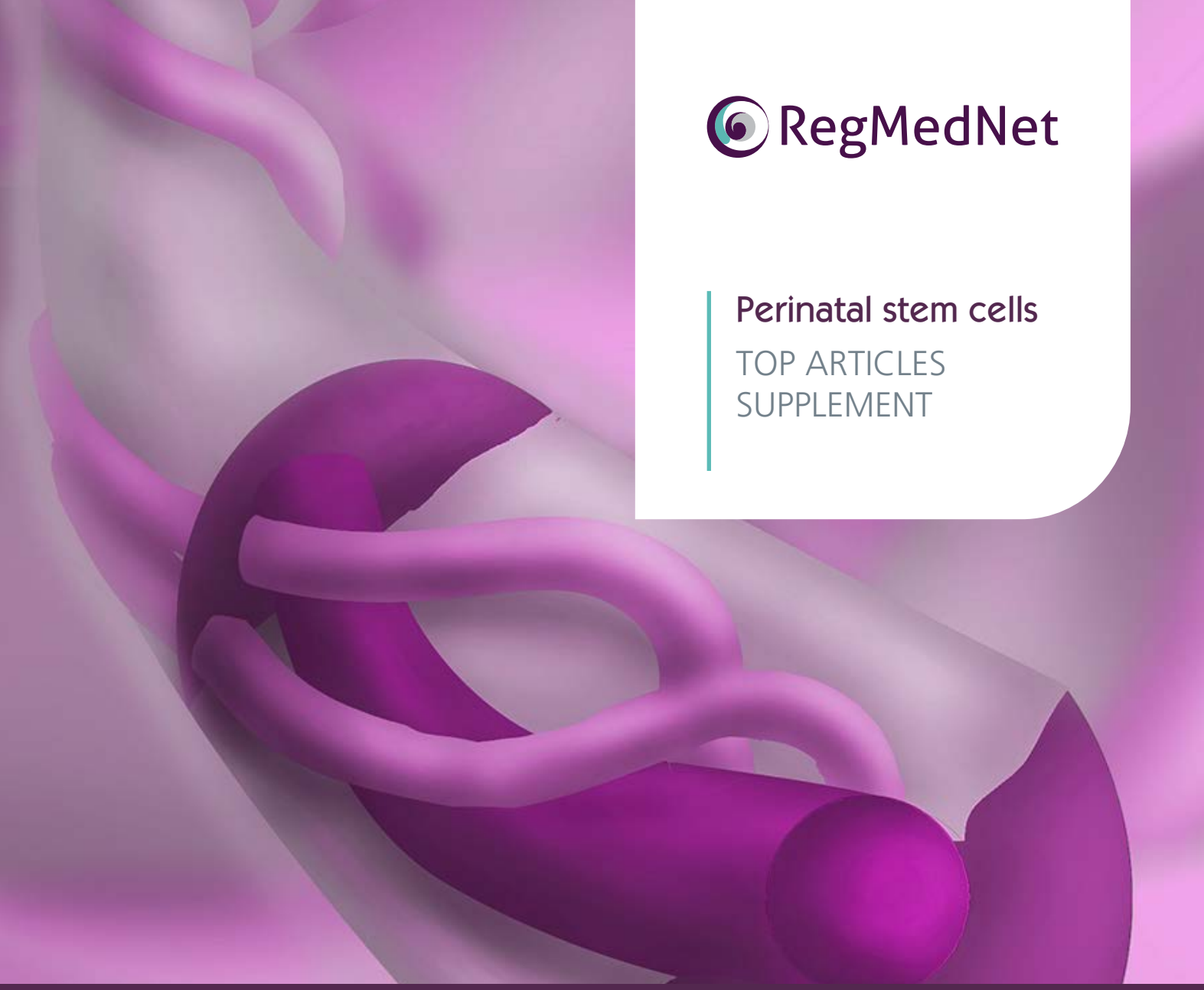

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Perinatal stem cells

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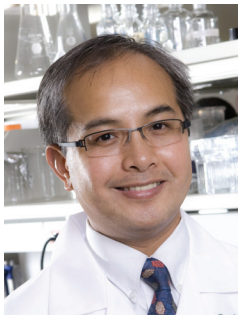
Stroke therapy: the potential of amniotic fluid-derived stem cells



"Exploring the potential of these cells [AFSCs] could lead to great advances in the fields of tissue engineering and regenerative medicine, and eventually toward the clinical application of these cells in stroke."

Maya Elias[‡,1], Jaclyn Hoover[‡,1], Hung Nguyen[‡,1], Stephanny Reyes[‡,1], Christopher Lawton[‡,1] & Cesar V Borlongan[*,1,2]

This article describes the clinical potential of cells harvested from human amniotic fluid in regenerative medicine, especially for stroke therapy. Amniotic fluid has been investigated as a new cell source for mesenchymal stem cells in the development of future cell-based transplantation. In this paper we highlight the characteristics of amniotic fluid-derived cells, as well as the functional benefits of these cells in animal models of stroke, altogether supporting the utility of amniotic fluid as stem cell source for cell therapy in stroke. The human amnion and amniotic fluid have attracted attention in recent years as possible reserves of stem cells that may have clinical application in the field of regenerative medicine. Several studies have examined the differentiation potential of cells derived from these two sources and concluded that they display high plasticity [1]. Currently, most studies have focused on cells derived from the amnion and revealed that amnion cell transplantation promotes re-epithelialization, modulates differentiation and angiogenesis and decreases inflammation, apoptosis and fibrosis [1–4]. This paper seeks to highlight what is currently known of the lesser studied, amniotic fluid-derived stem cells (AFSCs), and to acknowledge their potential clinical application for stroke therapy. Additionally, we will compare the advantages and disadvantages of amniotic fluid versus amnion membrane stem cells.

KEYWORDS
• placenta • regenerative medicine
• stem cells • tissue engineering
• transplantation

Stemness of cells derived from the amniotic fluid

The expression of specific pluripotency markers and genes in cells harvested from the amniotic fluid characterize these cells as stem cells. In a study by Antonucci *et al.* [5], molecular analysis of human second trimester AFSCs was found to express Fragilis, Stella, Vasa, c-Kit, and Rnf17, genes involved in early stages of germ cell development, while

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also expressing OCT4 and SOX2, markers of pluripotency. When AFSCs are aggregated to form embryoid bodies (EBs), they reacquire pluripotency potential and features of early stage embryogenesis that are usually lost [5]. Moreover, cells from AFSC-derived EBs express alternate spliced exons specific of pluripotent stem cells, such as the exon 10 of *DNMT3B* and the b isoform of *Sall4*; express markers of the three embryonic germ layers, such as *GATA4, GATA6, AFP* and *Nestin*; and there appears to be an absence of the X chromosome inactivation [5]. This observation further supports the important role of AFSCs in embryogenesis, as the reactivation of the inactive X chromosome may be correlated with genomic reprogramming events [5]. CD117-negative populations of human amniotic fluid mesenchymal stromal cells (AFMSCs) are readily abundant and can be easily reprogrammed into induced pluripotent stem cells (iPSCs) using nonintegrating Sendai viral vectors encoding for OCT4, SOX2, KLF4 and cMYC [6]. Furthermore, Jiang *et al.* [6] have demonstrated that these iPSCs were virtually indistinguishable from human embryonic stem cells in multiple assays, could be used to generate a relatively homogeneous population of neural progenitors, and show engraftment potential *in vivo. In vitro*, these neural progenitor cells were shown to be capable of differentiating into mature neurons and astrocytes [6].

AFMSCs also possess gene expression profiles that are largely characteristic of undifferentiated cells [7]. In a similar study by Antonucci *et al.* [7], RT-PCR analysis showed that AFMSCs express genes for Rex-1, SCF, GATA-4, vimentin, CK18, HLA ABC and FGF-5 throughout the culture period, and they express genes for BMP-4, nestin, AFP and HNF-4α. As these genes regulate a multitude of different cell types, these observations suggest that AFMSCs are able to differentiate into adipocytes, osteocytes, chondrocytes and neuronal cells; can express many pluripotent stem cell specific genes; and proliferate well during *ex vivo* expansion [7].

While AFSCs have raised much interest because of their ability to differentiate *in vitro* into lineages belonging to all three germ layers, their immune properties are still being assessed. AFMSCs have been regarded as cells with low immunogenicity. Studies have observed AFMSCs to be resistant to rejection because they express immunosuppressive factors such as CD59 (protectin) and HLA-G [8]. CD59 inhibits the complement membrane attack complex by binding C5b678 and hampering C9 from binding and polymerizing, thus preventing complement from damaging cells [8]. HLA-G, which is expressed in the placenta unlike HLA-A and HLA-B genes, plays a key role in immune tolerance in pregnancy, [8]. Other recent studies have shown immunomodulatory properties of AFMSCs, which can inhibit the proliferation of T lymphocytes [8]. In another study, cultured AFSCs demonstrated an increase in CD105+ cells in the late-passage compared with the early-passage AFSC cultures [9]. Because CD105 is a mesenchymal marker and the long-term culture conditions allowed mesenchymal cell growth, AFSCs have been suggested to be mesenchymal precursors [9]. Recent *in vitro* analysis has found that AFSCs modulate lymphocyte proliferation in different manners according to gestational age (i.e., those derived from first-, second- or third-trimester) [10]. Interestingly, first-trimester AFSCs significantly inhibited T cell and natural killer cell proliferation, while second- and third-trimester AFSCs were less efficient, and only inflammatory-primed second-trimester AFSCs could suppress B-cell proliferation [10].

The preceding studies demonstrate that AFSCs exhibit characteristics of both embryonic and adult stem cells and vary from donor to donor [11]. Moreover, protein expression in the cell types found in AFMSCs does not affect the differentiation capacity of AFMSC preparations (PMID: 25608581), and the ectopic expression of Oct-4 in hAFMSCs could be an alternative method to produce pluripotency [12], while the selective expression of SOX9 and induction of Wnt signaling can be used to specifically differentiate cells to neurons and promote neurogenesis, respectively [13,14]. However, before these methodologies can be used, it is important to identify a suitable cryopreservation protocol, such as the slow-freezing solution [15]. In a recent study, Zong *et al.* has shown to direct AFSCs to differentiate into neurons with characteristics of functionality using inner stem cells derived as a feeder layer [16]. The study also showed that the Wnt signaling pathway plays an important role in triggering neurogenesis [16]. Thus, with their multifaceted properties, these cells have an important application in stroke therapy.

Transplantation studies using amniotic fluid stem cells for stroke therapy (Maya)

As of 2010, stroke is the fourth-leading cause of death among adults in the USA, accounting for about one of every 19 deaths [17]. Currently, the only nationally approved treatment for acute ischemic stroke is intravenous recombinant tissue plasminogen activator, a thrombolytic, within a narrow 3-h window of symptom onset. Thrombolytic therapy has been shown to significantly reduce the proportion of deaths and dependence in activities of daily living [18]. However, thrombolytic therapy also carries an increase in the risk of death within the first 7–10 days, intracranial hemorrhage and death at a 3- to 6-month follow-up [18]. Intravenous delivery of bone marrow- and perinatal-derived cells, which have the capacity to translocate to areas of tissue injury and target brain remodeling, may be a therapeutic intervention within the first week after stroke, during the restorative phase [19]. Stroke is a time-limited acute injury; the brain may be more favorable to transplantation, than in other organ or systemic diseases characterized by ongoing degenerative processes or immunological attacks [20].

Neurons are derived from the ectoderm lineage. Previous studies have demonstrated the ability of amniotic fluid-derived stem cells to differentiate along a neurogenic pathway [21,22]. Transplantation of cells derived from the amniotic fluid has been explored in neurological disorders [7,23–25]. Transplantation of AFSCs may serve as a promising option for stroke therapy; however, there are only a few studies that focus on these cells as a source for transplantation in stroke. The goals of AFSC transplantation post-stroke are to promote restorative mechanisms, such as neurogenesis, angiogenesis and immunomodulation, and to contribute to functional improvement [20].

One study had investigated the effect of AFSCs on focal cerebral ischemia-reperfusion injury and its consequential behavioral deficits in a mouse model. This study employed middle cerebral artery occlusion for 60 min, followed by a reperfusion phase for 7 days, to produce this injury. Intracerebroventricular administration of amniotic fluid derived stem cells significantly reduced the neurological sequelae and behavioral deficits. The investigators also concluded that the beneficial effects of the AFSCs are comparable to those of embryonic neuronal stem cells, which typically carry ethical concerns [26].

Preclinical data of AFSC safety and efficacy suggests that individuals who suffer a stroke and show significant inflammation of the brain, or who display short-term memory loss due to the accompanying injury to the hippocampus, may be ideal candidates for future clinical trials of AFSC transplantation [27]. In addition, clinical trials of AFSC transplantation could be extended to patients with stroke of cardiovascular etiology, as preclinical data show substantial improvement in cardiac function following AFSC transplantation, suggesting that these cells are cardioprotective [28]. The administration route of AFSC transplantation may be dictated by the phase of stroke, that is, acute or chronic. Moreover, cryopreserved cells will also be required to ensure the ready availability of AFSCs for transplantation in both acute and chronic phases of stroke [27].

Our laboratory has tested the effect of amniotic fluid stem cell treatment in cerebral ischemia–reperfusion injury after stroke in rats [29]. Each rat was subjected to a series of behavioral tests to reveal neurological abilities prior to MCA occlusion, post-MCA occlusion and following transplantation of amniotic fluid-derived cells at day 35. Behavioral tests included the elevated body swing test and the rotarod test. We concluded that AFSC transplantation attenuates deficits in memory and learning, decreases infarct volume and neuron loss and increases cell proliferation [29].

Advantages & disadvantages of amniotic fluid versus amnion membrane stem cells

Both AFSCs and amnion membrane-derived stem cells (AMSCs) have their own advantages and disadvantages. **Figure 1** summarizes the advantages and disadvantages of the two sources of stem cells. First, amniotic fluid can be collected during amniocentesis, while the amnion membrane can only be harvested after childbirth. This is a critical difference between the two sources. The early harvest of amniotic fluid allows the AFSCs to be isolated, cultured and amplified prior to childbirth. Thus, if the child develops any disease during or immediately after delivery, such as hypoxia, then the baby's own stem cells are readily available for use. On the other hand, it takes weeks after delivery to amplify AMSCs from amnion membrane. The critical therapeutic window might have passed when ample supply of stem cells from amnion membrane is available for transplantation. Second, autologous

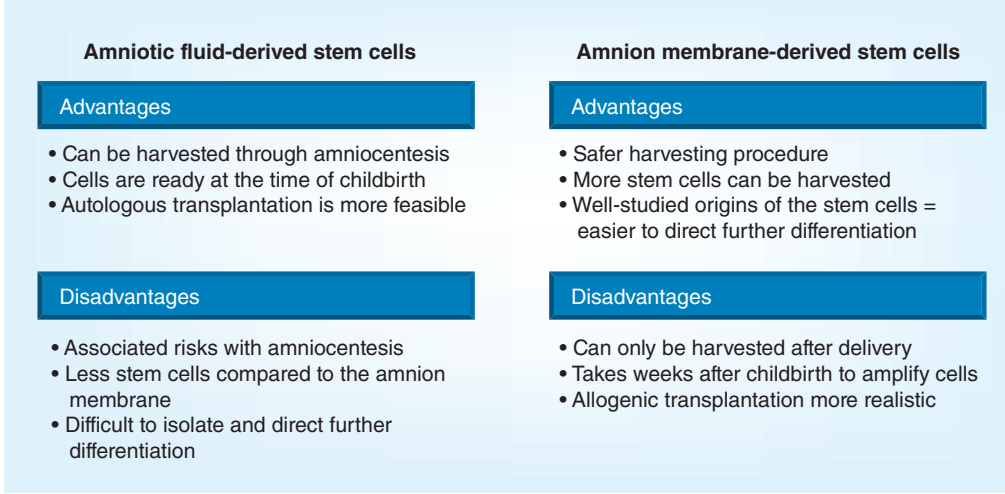

Figure 1. Advantages and disadvantages of amniotic fluid-derived stem cells and amnion membrane-derived stem cells.

transplantation is more feasible with amniotic fluid than amniotic membrane due to the former's early harvesting period (i.e., during amniocentesis). Hence the child can benefit from his/her own stem cells. With amniotic membrane, allogeneic transplantation is more realistic than autologous, again due to the time required to generate sufficient amount of stem cells. Third, safety issues with amnion fluid and membrane need to be considered. With AFSCs requiring amniocentesis, there may be risks associated with such procedure that can cause injury to the mother and/or the child. In contrast, AMSCs can be easily collected after childbirth, posing no harm to the child and the mother. For this reason, harvesting stem cells from the amnion membrane is a much safer procedure compared with amniotic fluid collection. Alternatively, the amniotic fluid can also be collected after childbirth, but the advantages mentioned above (e.g., readily available autologous AFSCs for transplantation during childbirth) are diminished. Fourth, the amnion membrane contains more stem cells compared with the amniotic fluid. Therefore it is easier to culture and amplify stem cells from amnion membrane than stem cells from amniotic fluid. However, because the amniotic fluid can be collected earlier using amniocentesis, there is plenty of time to amplify even with a smaller initial number of stem cells. Finally, it is difficult to isolate and confirm the stem cells' lineage of the amniotic fluid. AFSCs have to be phenotypically characterized to obtain a homogeneous cell population. Nonetheless, AFSCs have been shown to differentiate into multiple lineages [5,30–32]. On the other hand, the origin of amnion membrane is well studied. Most of the stem cells from amnion membrane are of epithelial and mesenchymal origins [33]. Hence, it is easier to isolate and direct further differentiation with AMSCs. However, recent studies have shown that most of the therapeutic effects of stem cells are from the secreted trophic factors, rather than the regenerated/differentiated stem cells [25,34]. Thus, a homogenous cell population may not be required as long as therapeutic outcomes are achieved.

Tissue engineering & regenerative potential of amniotic fluid stem cells

There is a possibility of harnessing the therapeutic properties of AFSCs and AFMSCs and applying them to tissue engineering and regenerative medicine for stroke. Studies from a relevant neurological disorder, traumatic brain injury (TBI), suggest the potential of AFSCs/AFMSCs as subdural patch-like network called biobridges. Biobridge formation may be fostered by AFMSC, as seen in notch-induced human bone marrow derived mesenchymal stromal cells during regeneration in a rat TBI model [35]. The formation of biobridges allows movement of exogenous and endogenous stem cells across non-neurogenic tissue to the site of injury [35]. This novel biobridge-based tissue engineering, coupled with the ability of AFMSC to differentiate into neural progenitor cells, may suppress the damaging inflammation. Moreover, the graft-host integration

following the biobridge formation occurs without eliciting an immune response, advancing the notion of an amniotic fluid subdural patch as a promising therapeutic agent for regenerative medicine.

Xenografts have become a potential treatment for many health disorders, including neurological diseases. However, cells, tissues and organs transplanted from one species to another are, in many cases, rejected by the host's immune system. Xenoreactive antibodies lead to complement activation and systemic inflammation, rejecting the xenograft immediately [36,37]. Even immune tolerance techniques such as neonatal desensitization have failed to improve cell survival [38]. However, previous studies using circulating anti-inflammatory alpha-1-antitrypsin (ATT) in combination with anti-CD4/CD8 therapy have shown to protect xenografts from inflammation, and as a result, promote xenograft acceptance [39]. With this in mind, AFMSC could be used as a new strategy to augment xenograft rejection. Co-transplantation of immunosuppressive amniotic fluid stem cells may then augment xenograft rejection.

In addition to dampening the immune and inflammatory response associated with xenografts, AFMSCs may also enhance the therapeutic outcome of allograft transplantation. Allograft tolerance can be described as the absence of a destructive reaction by the host's immune system against alloantigens of the transplant. The early phase of the immune response in allograft rejection is the innate and non-specific immune response. The subsequent phase is the donor-specific adaptive immune response, which results from T-lymphocyte recognition of the alloantigens [40]. The immunomodulatory properties of AFMSCs may help to promote long-term allograft acceptance and reduce chronic immunosuppression [8,41].

Conclusion

Although more is known about cells derived from the amniotic membrane than from amniotic fluid, there is great potential for future clinical application of AFSCs. The routine access to the amniotic fluid either during amniocentesis or after birth, easy isolation and amplification of stem cells, ability to differentiate into many cell lines, capacity to exert immunomodulatory effects and lack of ethical barriers associated with AFSCs make them an ideal cell source. AFSC transplantation could be utilized in the treatment of ischemic stroke, by promoting neurogenesis, angiogenesis and immunomodulation. Further research needs to be done to determine the full therapeutic spectrum of AFSC, along with identifying the optimal timing and best administration route for transplantation in clinically relevant stroke models. Exploring the potential of these cells could lead to great advances in the fields of tissue engineering and regenerative medicine, and eventually toward the clinical application of these cells in stroke.

Financial & competing interests disclosure

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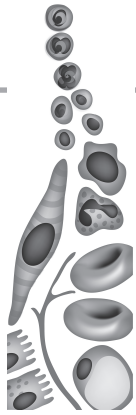

Assessment of umbilical cord tissue as a source of mesenchymal stem cell/endothelial cell mixtures for bone regeneration

Aim: To enumerate and characterize mesenchymal stem cells (MSCs) and endothelial cells (ECs) in umbilical cord (UC) tissue digests. **Materials & methods:** Cultured UC cells were characterized phenotypically, and functionally by using 48-gene arrays. Native MSCs and ECs were enumerated using flow cytometry. **Results:** Compared with bone marrow (BM) MSCs, UC MSCs displayed significantly lower (range 4–240-fold) basal levels of bone-related transcripts, but their phenotypes were similar ($CD73^+$, $CD105^+$, $CD90^+$, $CD45^-$ and $CD31^-$). UC MSCs responded well to osteogenic induction, but day 21 postinduction levels remained below those achieved by BM MSCs. The total yield of native UC MSCs ($CD90^+$, $CD45^-$ and $CD235\alpha^-$) and ECs ($CD31^+$, $CD45^-$ and $CD235\alpha^-$) exceeded 150 and 15 million cells/donation, respectively. Both UC MSCs and ECs expressed CD146. **Conclusion:** While BM MSCs are more predisposed to osteogenesis, UC tissue harbors large numbers of MSCs and ECs; such minimally manipulated 'off-the-shelf' cellular mixtures can be used for regenerating bone in patients with compromised vascular supply.

KEYWORDS: bone marrow ■ bone regeneration ■ endothelial cells ■ mesenchymal stem cells ■ umbilical cord

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Bone regeneration following fracture or tumor resection is highly challenging, and bone grafting procedures remain the gold standard therapeutic intervention [1]. Autologous grafts, however, possess numerous disadvantages including donor site morbidity and associated pain [2]. Bone marrow (BM) mesenchymal stem cells (MSCs) were first used in combination with osteoconductive scaffolds to repair bone [3,4]. Additionally, systemic administration of BM MSCs has been used in both autologous and allogeneic settings to treat osteogenesis imperfecta [5]. BM MSCs were also the first type of MSCs shown to possess immunoregulatory capacity [6], leading to worldwide therapeutic use for the treatment of graft-versus-host disease [7].

BM MSCs are relatively easy to procure, although their rarity in BM aspirates (0.001–0.01%) necessitates culture amplification in order to achieve the required number of cells for therapy [8]. However, extended MSC passaging is to be avoided owing to the loss of potency and concerns relating to a potential for accumulating senescent cells and chromosomal abnormalities [9,10]. With these issues in mind, MSCs of perinatal origin have attracted increased interest as potential candidates for bone repair applications. Umbilical cord (UC) tissue, UC blood, fetal liver, villous placenta, fetal membranes and amniotic fluid have all been shown to host MSCs [11–14]. Human UC tissue in particular represents an attractive MSC source for bone regeneration [15]; this is not only because their harvest is noninvasive, but also because of their juvenile biological age, which argues for a lower possibility of genetic alterations [14]. However, controversy still exists regarding the osteogenic capacity of UC MSCs in comparison to BM MSCs. Early studies showed that UC MSCs could in principle differentiate towards osteoblasts [16–19], however, later studies documented that UC MSCs were only weakly osteogenic [20,21]. These discrepancies could be explained by the fact that UC MSC cultures in the aforementioned studies were derived from different locations within the UC tissue [22]. The inner tissue architecture of UC is comprised of two arteries and one vein, which are surrounded by a matrix of mucous connective tissue, termed Wharton's jelly [23]. MSCs derived from perivascular and Wharton's jelly areas, in particular, may have different propencities for osteogenesis [18,20].

It should be kept in mind that bone formation *in vivo* is mediated by MSCs that closely interact with local vasculature [24]; and normally, endothelial cells (ECs) crosstalk with adjacent MSCs in their periendothelial niche [25,26], and influence MSC behavior *in situ*. In physiological bone repair processes, newly synthesized vessels are stabilized by MSCs and anastomose with pre-existing vessels, therefore long-lasting, functional regeneration of truly

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vascularized bone is now believed to require both cell sources [27–29]. Minimally manipulated BM isolates, containing autologous MSCs and potentially ECs, have been successfully used in orthopedic settings [30,31]. The advantage of using UC tissue in this context would be the coexistence of both MSCs and large numbers of ECs [32] within the same tissue.

The aim of this study was to compare the *in vitro* osteogenesis of UC MSCs with that of BM MSCs at functional and transcriptional levels. Our secondary aim was to enumerate MSCs and ECs in UC digests in comparison to BM aspirates. In summary, we show that UC MSCs are inferior to BM MSCs in their *in vitro* osteogenic capacity, underscored by their tissue-specific molecular signatures. However, our enumeration experiments revealed that the yields of native MSCs ($CD45^-$, $CD90^+$ and $CD235\alpha^-$ cells) and native ECs ($CD45^-$, $CD31^+$ and $CD235\alpha^-$) from a single UC donation considerably exceeded those obtained from an average 20-ml specimen of BM aspirate (average 90- and 11-fold, respectively). The simultaneous isolation of UC MSCs and ECs could therefore provide a novel means of generating a large pool of native regenerative cells for allogeneic bone repair applications.

Materials & methods

Cell processing

Cells were isolated from 19 UCs of full-term elective cesarean section patients. The whole UC tissue was mechanical sliced into small pieces (approximately 0.2 g) and washed repeatedly with phosphate-buffered saline (Life Technologies, UK). Single-cell suspensions were liberated using 6-h incubation with 600 U/ml collagenase I (Stem Cell Technologies, France) at 37°C with gentle rotation. The average cellularity of enzymatically released UC isolates was 7.5×10^7 nucleated cells/per gram of tissue. BM aspirates were obtained from the iliac crest of ten healthy individuals (median age 45 years; range: 7–85). BM mononuclear cells (MNCs) were isolated using Lymphoprep (Axis-Shield, UK) density gradient centrifugation and the average cellularity of BM MNC fractions was 3.1×10^7 cells/ml. Sample collection and the study protocol were approved by Leeds Teaching Hospitals Research Ethics committee (UK).

Expansion of MSC & EC cultures

For primary MSC culture, cells isolated from UC digests and BM aspirates were seeded into six-well cluster plates (Corning) in nonhematopoietic, MSC-specific medium (Miltenyi Biotec) at the cell seeding density of 5×10^4 cells/well (approximately 5×10^3 cells/cm^2). After observing colony formation and subsequent cell 80% confluency (denoted passage 0 [p0]), adherent cells were trypsinized using trypsin/EDTA (Life Technologies), reseeded and serially passaged (for at least a further 40 days). The number of viable cells at each passage was recorded and the number of population doublings (PDs) between passages ([p] starting from p0) was calculated according to the following equation: PD = log2 (Nt/Ni), where Nt and Ni are the terminal and initial cell numbers, respectively. PDs before p0 were calculated based on the colony-forming unit-fibroblast (CFU-F) potential of cells seeded to initiate cultures and the number of cells at p0 according to the equation PD = log2 (number of cells at p0/number of seeded CFU-Fs). For primary culture of ECs, digested cells were seeded in six-well plates (4000 cells/cm^2) coated with 2 µg/cm^2 fibronectin (BD Biosciences, UK), to allow for specific attachment of ECs, and grown in EBM-2 medium (Lonza, UK). The EBM-2 medium contains fetal bovine serum, epithelial growth factor, vascular endothelial growth factor, fibroblast growth factor-basic, insulin-like growth factor, hydrocortisone, heparin, ascorbic acid and antibiotics, and is designed to support EC growth [33]. On day 2, adherent cells were washed in phosphate-buffered saline and similarly passaged for further 40 days; at p1 of culture no contaminating cells were evident by flow cytometry. The isolation efficiency of both MSCs and ECs was 100% (no donor failed to generate cultures). Altogether, we generated six donor-derived UC and BM MSC cultures each, and three individual EC cultures; no pooled cultures were used.

Differentiation assays

p3-cultured MSCs were subjected to standard differentiation induction protocols, as previously described in our laboratory [34]. In brief, osteogenic differentiation was induced in medium containing low-glucose DMEM (Invitrogen, UK), 10% fetal calf serum, 100 µM ascorbic-2-phosphate, 10 mM β-glycerophosphate and 100 nM dexamethasone (all from Sigma-Aldrich, UK), and assessed using staining for alkaline phosphatase, Alizarin Red (both from Sigma-Aldrich) or by measuring calcium deposition [35]. Chondrogenic differentiation was performed on pellet cultures followed by staining with Toluidine blue (Sigma-Aldrich) and measurement of sGAG deposition [35]. Adipogenic

differentiation was assessed by staining with 0.5% Oil Red (Sigma-Aldrich) [35].

Flow cytometry on cultured cells

Phenotypic characterization of cultured MSCs and ECs was performed at early and late passages (<20 and >20 PDs, respectively) to evaluate the expression of markers specific to MSC, hematopoietic and endothelial lineages. The antibodies used were: CD31-FITC, CD105-PE, CD90-PE (Serotec, UK), CD45-FITC, CD34-PE (DAKO), CD73-PE, CD146-PE (BD Pharmingen, UK) and CD271-PE (Miltenyi Biotec, UK). Three-color flow cytometry was performed using FACScan (BD), as previously described using 2 µg/ml propidium iodide (Sigma-Aldrich) for dead cell exclusion [34]. Isotype controls were from Serotec. All flow cytometry analysis was performed using CellQuest software version 3.1 (BD Biosciences).

Flow cytometry on uncultured cell isolates

Evaluation of MSC and EC populations in UC tissue digests or BM MNC fractions was assessed using six-color flow cytometry on a BD LSRII flow cytometer. Per sample, 2×10^6 freshly isolated cells were stained with either CD90-PE or CD146-PE, and CD31-APC (Miltenyi Biotec) in combination with CD45-PECy7 (BD Pharmingen) and CD235a-FITC (Dako), the latter two to evaluate leukocytes and erythroid-lineage cells, respectively. Dead cells were excluded using 2 µg/ml 7-aminoactinomycin D (7-AAD; Sigma-Aldrich). Data acquisition and analysis were performed using FACS Diva (BD Biosciences) and the proportions of different cell fractions were calculated as percentages of total live (7-AAD negative) cells. It should be noted that International Society for Cellular Therapy (ISCT) criteria, with the addition of novel markers, have been followed for the phenotypic characterization of cultured MSCs. However, the ISCT panel was too broad to allow its application on freshly digested tissues; the latter phenotyping required the 'gating out' of dead cells, hematopoietic and endothelial lineage cells, excluding three fluorescence channels on the flow cytometer. Therefore, $CD90^+$, $CD45^-$ and $CD31^-$ strategy was used to identify MSCs in uncultured cell isolates.

Gene expression studies using MSCs & ECs

Taqman low-density arrays (TLDAs) were used to compare transcript levels in both MSCs and ECs. RNA was isolated from cells using RNA/DNA/Protein purification kit (Norgen) and cDNA produced using Superscript II (Life Technologies). A format 48-TLDA (Applied Biosystems) was used and included genes involved in osteogenic, chondrogenic, adipogenic, angiogenic and myogenic lineage differentiation, pluripotency, as well as novel osteogenically related transcripts recently discovered in our laboratory [36]. The full list of 48 separate transcripts is shown in TABLE 1 & SUPPLEMENTARY TABLE 1 (see online at www.futuremedicine.com/doi/suppl/10.2217/RME.13.47). The TLDAs were run according to the manufacturer's protocol, using 100 ng cDNA. All the expression data were obtained using the ABI PRISM® 7900HT sequence detection system. Values for each transcript were normalized to *HPRT1* (ΔCt) and relative expression was calculated using the formula $2^{-\Delta Ct}$. Gene cluster analysis was performed as previously described [36].

Data analysis

To identify differences between the three study groups tested (BM MSCs, UC MSCs and UC ECs), TLDA results were analyzed using the Kruskal–Wallis test with Dunn's correction for multiple group comparisons. The nonparametric Mann–Whitney test was used to compare independent samples. p-values less than 0.05 were considered significant. Cluster analysis of gene expression data was performed as previously described [36].

Results

Differentiation potentials of UC & BM MSCs

Trilineage mesenchymal differentiation potentials of UC and BM MSCs were investigated using p3 cultures (corresponding to approximately 15 cumulative PDs). Alkaline phosphatase activity by osteogenically-driven UC MSCs appeared visually weaker compared with BM MSCs (FIGURE 1A, far left panel) and their calcium production on day 21 postinduction was significantly lower ($p < 0.05$; FIGURE 1A, far right panel). Chondrogenic differentiation capacity of UC MSCs was donor-variable but on average similarly inferior compared with that of BM MSCs (FIGURE 1A, middle left panel, average 15 vs 1 µg/pellet, respectively, $p < 0.05$; $n = 3$ in each group). UC MSCs displayed lower lipid accumulation than BM MSCs in adipogenic conditions (FIGURE 1A, middle right panel), as shown by us recently using a quantitative assay using Nile red [37].

Next, MSC osteogenic differentiation was evaluated in a time-course study (FIGURE 1B). It

Table 1. List of differentially expressed genes in undifferentiated bone marrow and umbilical cord mesenchymal stem cells.

Assay ID	Description	Gene name	Fold difference BM:UC MSCs*
Hs00192325_m1	Osteomodulin	*OMD*	–†
Hs00154192_m1	Bone morphogenetic protein 2	*BMP2*	240
Hs00180066_m1	Secreted frizzled-related protein 4	*SFRP4*	50
Hs01005963_m1	Insulin-like growth factor 2	*IGF2*	29
Hs00269972_s1	CCAAT/enhancer-binding protein-α	*CEBPA*	9
Hs00610060_m1	Secreted frizzled-related protein 1	*SFRP1*	6.5
Hs00900058_m1	Vascular endothelial growth factor-α	*VEGFA*	6
Hs00998018_m1	Platelet-derived growth factor receptor-α	*PDGFRA*	5
Hs00173634_m1	Vascular endothelial growth factor-β	*VEGFB*	4
Hs01028971_m1	Collagen type 1 α 2	*COL1A2*	4
Hs01052937_m1	Vascular endothelial growth factor receptor 1	*FLT1*	0.03
Hs00403062_m1	BMP-binding endothelial regulator	*BMPER*	0.5

**p < 0.05, Mann–Whitney test. Fold changes were based on mean values.*
†The expression was below detection in UC MSCs, therefore no fold changes or statistical values could be calculated.
BM: Bone marrow; MSC: Mesenchymal stem cell; UC: Umbilical cord.

was previously suggested that osteogenic progression of UC MSCs proceeds at a faster rate compared with BM MSCs [18], with the peak of mineralization occurring earlier, before day 21 when it is commonly analyzed. By contrast, both staining for ALP activity (left panel) and Alizarin Red indicating mineralization (right panel) revealed the slower rate of osteogenesis in UC MSCs (Figure 1B) in our differentiation conditions.

Altogether, these data indicated that both BM and UC MSC cultures were tripotential, however, UC MSCs were inferior in their differentiation capacities, particularly with respect to osteogenesis, which proceeded much slower.

Growth & phenotypic characteristics of UC & BM MSCs

We next hypothesized that the lower osteogenic capacity of UC MSCs could be due to their lower levels of purity, due to potential contamination with other adherent cells (since whole UC tissue digests were used to initiate the cultures). However, p3 cultures from both sources expressed classic MSC markers CD73, CD90 and CD105 at similar levels (all >90% positive) and were negative for hematopoietic (CD45) or endothelial lineage (CD31) markers (n = 3 donors in each group; Figure 2A). Previous studies have documented other molecules (CD146 [38,39] or CD271 [34]) as potential MSC markers. CD146 was expressed on the majority (>80%) of p3 MSCs from both tissues and declined only slightly (to approximately 50–70% of cells) at late passages (>20 PDs; Figure 2A). CD271 was largely absent on both types of cultured MSCs, consistent with previous findings pertaining to cultured BM MSCs [34].

A possibility of contamination of UC MSC cultures with more mature adherent cells was assessed in long-term cultivation experiments (Figure 2B). UC MSCs grew fast (n = 5 donors tested) and did not show signs of senescence up to 60 days in culture, rejecting this possibility. These data indicated that the observed differences in osteogenesis between the two MSC types were unlikely to be a result of lower purity of the UC MSCs.

Transcriptional profile of UC & BM MSCs before osteogenic induction

We next reasoned that the observed functional differences in osteogenic capacities of UC and BM MSCs could be a result of different predisposition to osteogenesis at the RNA level. To investigate this, transcriptional profile analysis was performed using both types of MSCs (n = 4 and 3, respectively), and UC ECs (n = 3) were additionally employed as a negative/non-MSC control. UC ECs were generated from UC digests by attachment to fibronectin and specific expansion in EC-specific EBM-2 medium [37]. The majority of genes were selected based on their involvement in osteogenic–angiogenic interactions [40,41], including novel genes recently described in our laboratory [36].

Cluster analysis revealed a close relationship between UC and BM MSCs and a separate branching of UC ECs (Figure 3A). Compared with both types of MSCs, ECs overexpressed *FZD4*/frizzled 4, *LRP5*/low-density lipoprotein receptor-related protein 5 and *PECAM1*/CD31 (Figure 3B). In ECs, the CD31 transcript was expressed approximately 500-fold higher compared with both MSCs and, as expected, no CD31 protein expression was observed on the surface of MSCs by flow cytometry (Figure 3C). The transcripts for *PGF*/placental growth factor, *KDR*/VEGFR2 and *MCAM*/CD146 were significantly higher in ECs compared with UC MSCs (Figure 3B). At the surface protein level, CD146 was found to be similarly expressed in all three cell types (Figure 3C). Therefore, the lack of CD146 specificity for MSCs was demonstrated at both mRNA and protein levels.

Statistical analysis for two independent groups of MSCs (UC and BM) has revealed drastically lower expression of mature bone proteins *OMD*/osteomodulin and *COL1A2*, molecular agonists *BMP2* and *IGF2*/insulin-like growth factor 2, Wnt pathway regulators *SFRP4* and *SFRP1*, and transcripts for vascular endothelial growth factors *VEGFA* and *VEGFB* in UC MSCs ($p < 0.05$; Table 1). *FLT1*/vascular endothelial growth factor receptor 1 and *BMPER*/BMP binding endothelial regulator were upregulated in UC MSCs compared with BM MSCs (Table 1). Previous literature implicated *FLT1* upregulation with hypoxia-driven neovascularization and angiogenesis [42], whereas BMPER was shown to regulate bone morphogenetic protein-dependent angiogenesis [43]. Overall, the majority of osteogenesis-related molecules were expressed at higher levels in BM MSCs compared with UC MSCs.

Transcriptional profile of UC & BM MSCs following osteogenic differentiation

Next, the responsiveness of UC and BM MSCs to *in vitro* osteogenic induction was assessed using the same TLDA card. Changes in gene expression levels in differentiated cells on day 21 postinduction were compared with day 0 levels (i.e., levels in undifferentiated cells; n = 4 and n = 3 for UC and BM MSCs, respectively; Figure 4). In BM MSCs, the highest upregulation postosteoinduction (up to 100-fold) was observed for *FRZB*/*SFRP3* and *IGF2* (Figure 4A). These transcripts were also upregulated in UC MSCs (up to 80-fold), but their levels postinduction rarely reached the levels of differentiated BM MSCs.

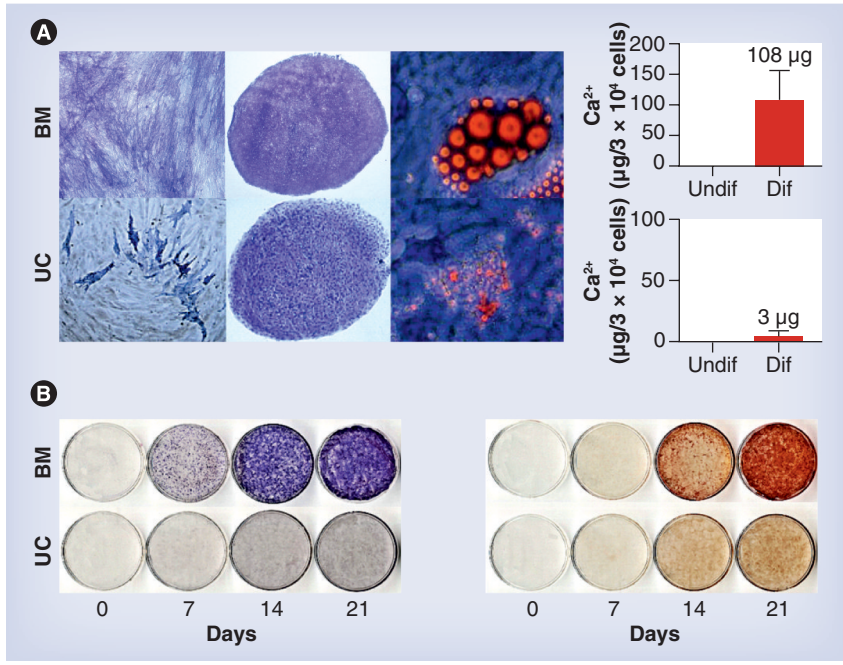

Figure 1. Differentation capacity of umbilical cord and bone marrow mesenchymal stem cell cultures. (A) Osteogenesis, chondrogenesis and adipogenesis (left panel; from left to right) and calcium (Ca^{+2}) production (right panel). Staining for alkaline phosphatase (day 14), toluidine blue (day 21) and Oil Red O (day 21), respectively. Original magnification: osteogenesis, ×100; chondrogenesis, ×40; and adipogenesis, ×200. Ca^{2+} production (µg/3 × 10^4) by BM mesenchymal stem cell (MSCs; n = 6) and UC MSCs (n = 6) was measured before (Undif) and after (Dif) osteoinduction; error bars represent standard deviations. **(B)** Time course of the osteogenic progression (representative donor). Alkaline phosphatase (blue) and Alizarin red (red) staining are weaker in UC MSCs at all time points studied.
BM: Bone marrow; Dif: Differentiated; UC: Umbilical cord; Undif: Undifferentiated.

Two other secreted frizzled-related proteins (SFRPs) under investigation were consistently upregulated in UC MSCs following osteoinduction, but at best, their induced levels reached only the basal levels of BM MSCs and were considerably less than in osteogenically differentiated BM MSCs (Figure 4B). Interestingly, angiogenesis-related transcripts *PGF* and *VEGFA* were consistently downregulated following osteogenic differentiation in BM MSCs, but not in UC MSCs (Figure 4C). In fact, not a single transcript displayed a consistent downregulation in its expression in osteogenically differentiated UC MSCs.

OMD, a mature bone protein transcript involved in osteoblast maturation, also displayed upregulation postosteoinduction in UC MSCs but due to undetectable levels in undifferentiated UC MSCs (Table 1) values/fold changes could not be calculated. Still, *OMD* transcript levels in differentiated UC MSCs did not reach the expression levels in differentiated BM MSCs.

Overall, these molecular profiles indicated that BM MSCs were more committed and

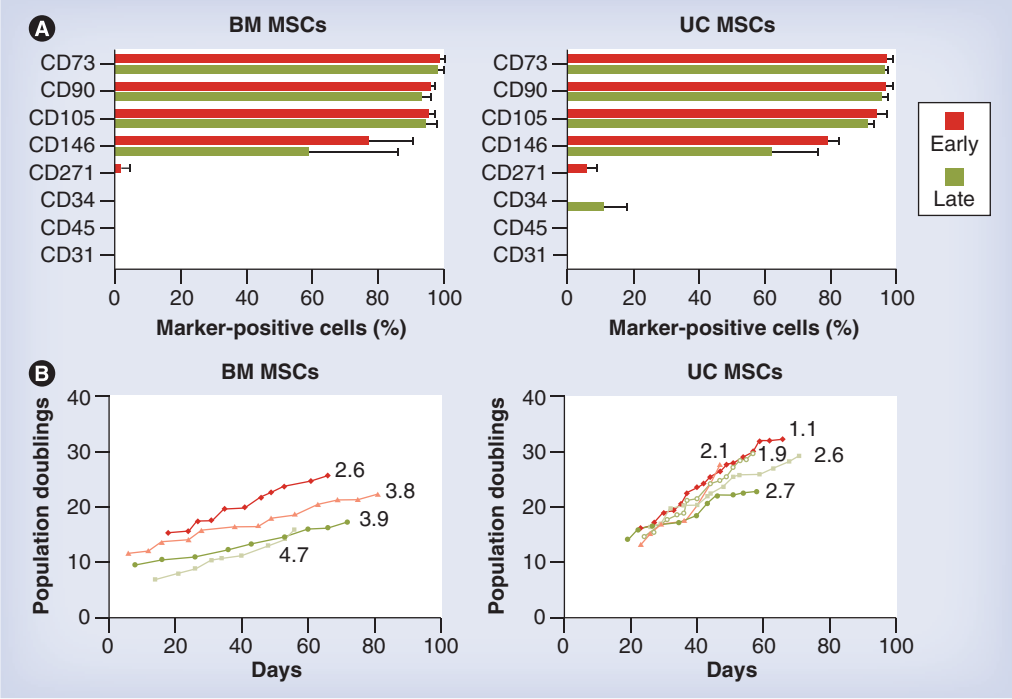

Figure 2. Phenotypic profiles and growth kinetics of umbilical cord and bone marrow mesenchymal stem cells. (A) Similar surface marker expression levels in early (<20 population doublings) and late cultures (>20 population doublings; n = 3 donors in each group, error bars represent standard deviations). **(B)** Growth kinetics of BM MSCs (four donors) and UC MSCs (five donors). Population doubling times (in days/population doubling) are shown for each donor (represented by different symbols).
BM: Bone marrow; MSC: Mesenchymal stem cell; UC: Umbilical cord.

responding well to osteogenic stimuli, whereas UC MSCs appeared to be responsive but unable to progress all the way to the same level of commitment as differentiated BM MSCs.

Enumeration of native MSCs & ECs in UC digests

Although UC MSCs had a lower predisposition to osteogenesis (Figure 3), they were still able to respond to osteogenesis stimulation (Figure 4). Therefore, we next explored whether the value of UC tissue for bone repair applications would be primarily as a source of large numbers of regenerative cells including ECs. For this, six-color flow cytometry was performed to identify and quantify native MSC and EC populations in UC digests compared with BM aspirates (n = 5 and n = 3 for UC and BM donors, respectively; Figure 5).

Despite extensive washing, the overwhelming majority of cells in both sources were $CD45^+$ hematopoietic cells (average 86 and 91% for UC digests and BM aspirates, respectively; Figure 5A). The proportions of $CD235\alpha^+$ erythroid precursors were similar and averaged approximately 1% (Figure 5A). These hematopoietic cell subsets were subsequently 'gated out', meaning removed from the analysis, and native MSCs and ECs were identified based on CD90 and CD31 expression, respectively. The frequency of native MSCs ($CD90^+$, $CD45^-$ and $CD235\alpha^-$) was significantly higher in UC digests compared with BM aspirates (approximately 70-fold; $p < 0.05$; Figure 5A). Native ECs ($CD31^+$, $CD45^-$ and $CD235\alpha^-$) were twofold more numerous in UC digests compared with BM aspirates (not significant; Figure 5A). Based on our flow cytometry data using whole UC tissue digests, the proposed yield of native MSCs and ECs from UC, based on CD90 and CD31 markers, respectively, could be as high as 151–170 and 15–17 million cells, respectively (taking into consideration the average weight of a human full-term UC of 40–45 g). Based on native cell frequencies in BM aspirates, such numbers of MSCs and ECs could not be achieved from a single BM donation (would require up to approximately 1.8 l for MSCs and 220 ml for ECs).

We next performed a more detailed phenotypic analysis of native UC ECs. The gating strategy (R1) and identification of a distinct native UC EC population (R2) are shown on Figure 5B. Native UC ECs lacked CD90, the

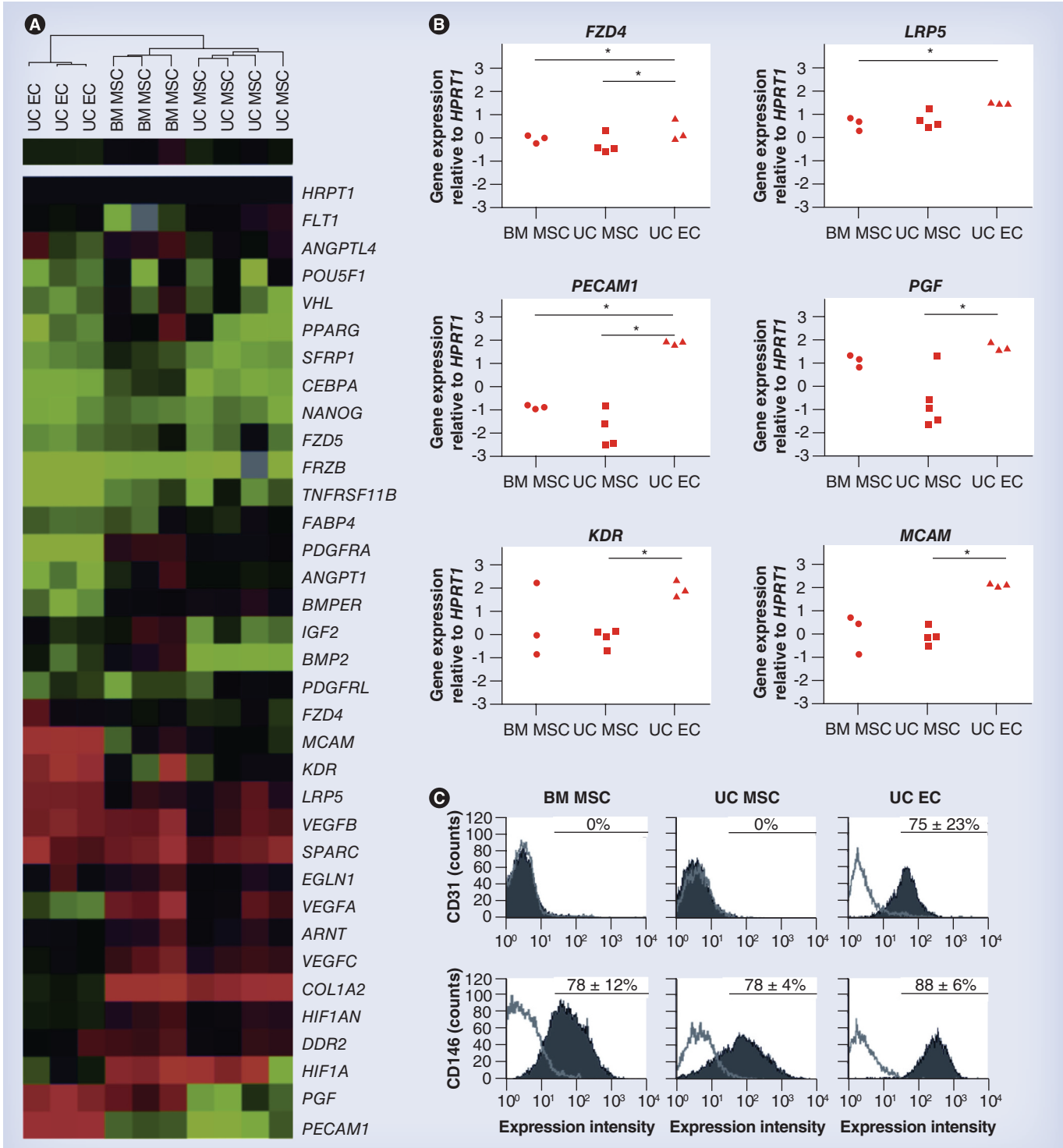

Figure 3. Transcriptional profiling of bone marrow mesenchymal stem cells, umbilical cord mesenchymal stem cells and umbilical cord endothelial cells by Taqman low-density arrays and selected transcript validation by flow cytometry. (A) The heat map showing distinct clustering of both MSC types away from ECs. Log_2 transformation was performed according to the methods described in the Cluster & TreeView Manual and scores were assigned as follows: red = >1, green = <1, black = 1 and grey = missing data (below detection). **(B)** Transcripts highly specific for ECs. Results for individual donors are shown. Y-axis represents transcript expression levels normalized to the *HPRT1* gene (scale log_{10}). **(C)** Flow cytometry validation of *PECAM1*/CD31 expression and *MCAM*/CD146 expression (filled histograms: marker; open histograms: isotype control). Horizontal lines represent the region used to calculate percentages of marker-positive cells; the data above the lines indicate average proportions of positive cells ± standard deviation (n = 3 donors).
*$p < 0.05$, Kruskal–Wallis test with Dunn's correction.
BM: Bone marrow; EC: Endothelial cell; MSC: Mesenchymal stem cell; UC: Umbilical cord.

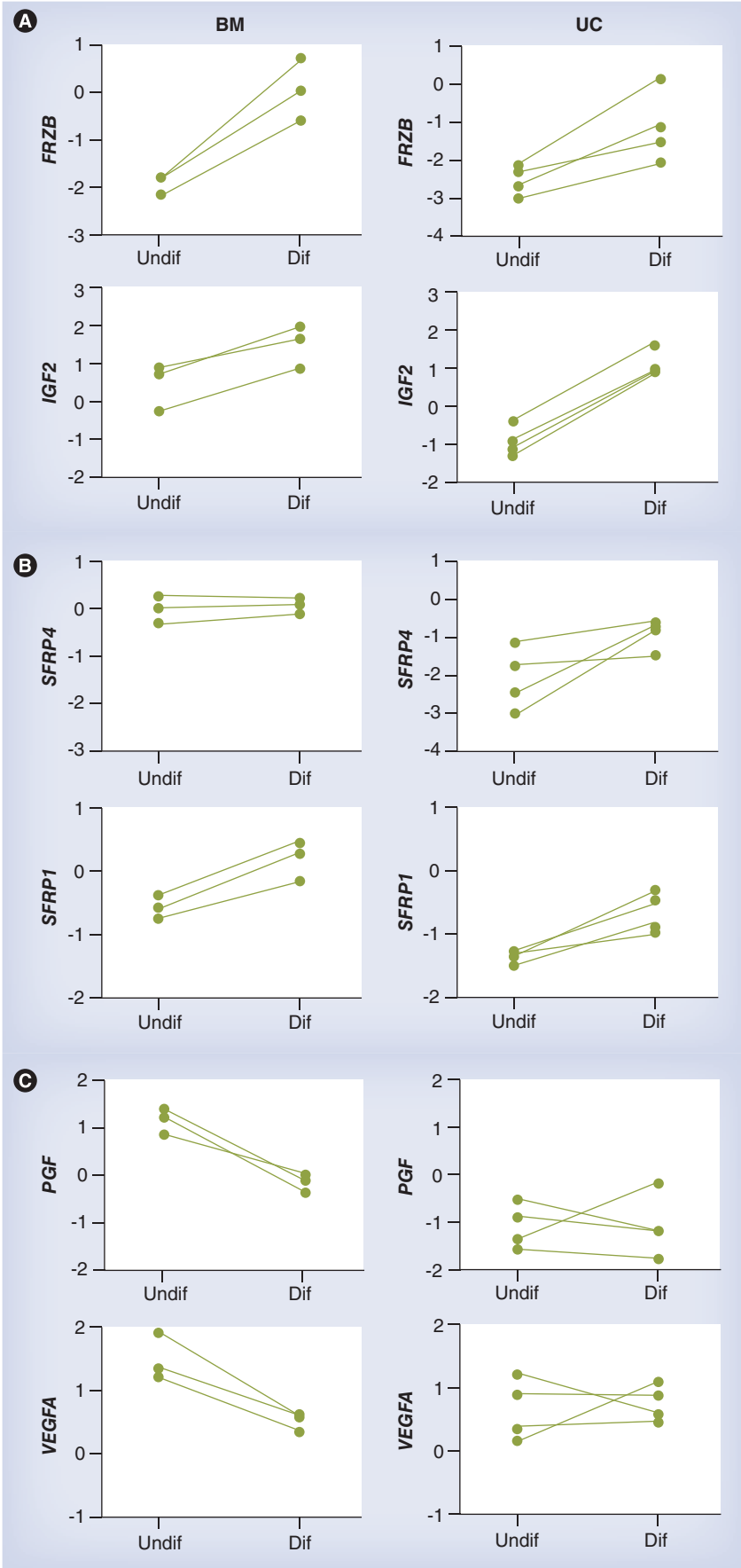

Figure 4. Preosteoinduction (undifferentiated) and postosteoinduction (differentiated) expression patterns of selected bone- and angiogenesis-related transcripts. (A) Two representative transcripts (*FRZB* and *IGF2)* that displayed strong and consistent upregulation in both types of mesenchymal stem cells (MSCs). **(B)** The expression of two Wnt pathway regulators *SFRP4* and *SFRP1* showing consistent upregulation in day 21 differentiated UC MSCs, although the levels of differentiated BM MSCs were never achieved. **(C)** Angiogenesis-related transcripts that were consistently downregulated in BM MSCs but not in UC MSCs, where they commonly remained expressed at comparably low levels (n = 4 donors for UC MSCs and n = 3 donors for BM MSCs, scale $\log_{10}$).
BM: Bone marrow; Dif: Differentiated; UC: Umbilical cord; Undif: Undifferentiated.

robust MSC marker, and 80 ± 16% of them expressed CD146 (Figure 5C). These data clearly indicated CD146 was expressed on native UC ECs and hence, contrary to recent publications pertaining its specificity for BM MSCs [25], CD146 was not solely specific for MSCs, at least in the UC tissue.

Discussion

Effective bone tissue regeneration depends on the correct combination of cells, scaffolds, growth factors and mechanical stimuli [44]. Recently, UC MSCs were proposed as a source of MSCs alternative to BM [15,45], not only because their isolation is noninvasive [22,46,47], but also because of their ability to rapidly colonize polymer scaffolds and demineralized bone matrices *in vivo* [45,48].

In this study, we demonstrated the inferiority of cultured UC MSCs over BM MSCs in standard *in vitro* differentiation conditions. We explored potential molecular mechanisms behind these observations and found that several transcripts related to osteogenesis were significantly lower in undifferentiated UC MSCs; this particular expression pattern could be responsible for the lower osteogenic capacity of UC MSCs observed *in vitro*. Our findings support a previously proposed concept that functional differences between MSCs from different sources could be linked to basal levels of gene expression (i.e., before osteogenic induction) [13,49].

Our findings are consistent with previous studies describing the lower osteogenic potency of UC tissue-derived MSCs [20,21]. The differences between our study and previous data, in which greater osteogenic capacity in UC MSCs

was observed [18,19], could be explained by the fact that in the latter studies UC MSCs were specifically derived from perivascular areas of UC tissue, the regions known to contain more mature progenitors compared with intervascular areas [22,50]. In further experiments, specialized 3D systems could be tested to better mimic bone formation *in vivo* and possibly enhance osteogenesis of UC MSCs.

Although MSCs resident in different tissues all fulfil the minimal criteria for MSCs [51], their degrees of differentiation capacity differ. For example, periosteal MSCs are better at osteogenesis compared with synovium-resident MSCs [49]. Synovial MSCs, on the other hand, possess better chondrogenesis compared with adipose- or BM-derived MSCs [52]. It is currently believed that differing propensities of MSCs towards a certain lineage are dictated by physiological demands for cell replacement within that tissue [49]. This is much harder to conceptualize for MSCs from perinatal tissues such as UC or placenta: recently shown to be similarly inferior in osteogenesis when compared with BM MSCs [53]. The study by Guillot *et al.* using fetal tissues (BM, blood and liver) has documented a hierarchy in osteogenesis, with fetal BM perhaps unsurprisingly showing the best osteogenic potential [13].

When comparing osteogenesis of MSCs derived from different tissue sources, only a few studies have attempted to address molecular mechanisms behind these observations. In many cases, certain surface markers were found to be differentially expressed and putatively linked with the observed functional differences. A recent study by Pilz *et al.* found an association between higher CD146 expression by BM MSCs and their stronger osteogenesis, compared with placenta-derived MSCs [53]. In many other studies, however, such links with MSC surface marker expression have not been found [49,54].

In the present study, UC and BM MSCs had a classic MSC phenotype and no quantitative differences in marker expression were found. Cluster analysis revealed distinct grouping of UC and BM MSCs away from ECs. Six out of 45 molecules, including CD146, were expressed at higher levels in ECs compared with MSCs. Compared with BM MSCs under normal culture-expansion conditions, UC MSCs expressed lower levels of ten transcripts whereas only two transcripts were found to be expressed at a greater level. Day 21 osteogenically differentiated BM MSCs displayed a consistent upregulation of osteogenesis-related transcripts, whereas some transcripts related

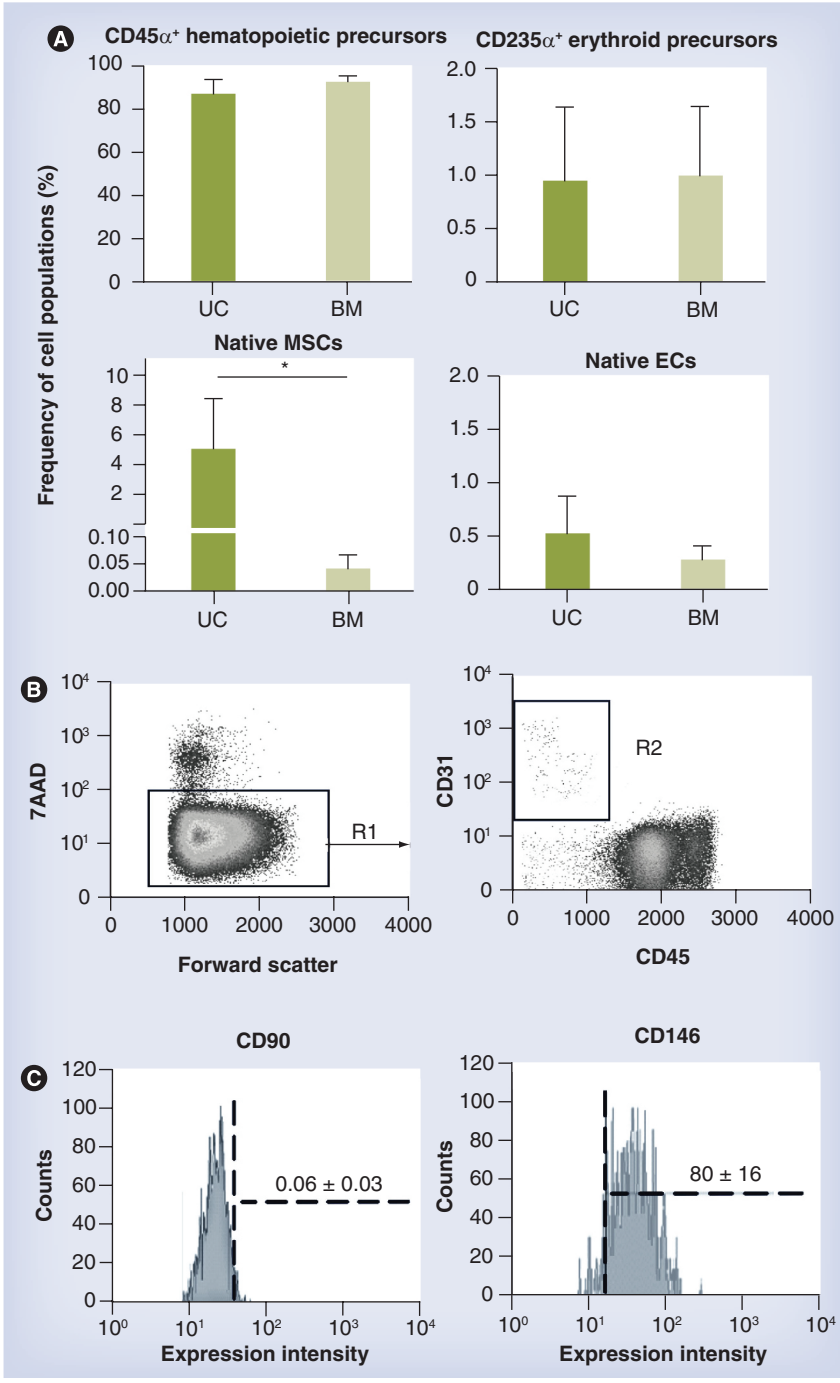

Figure 5. Cellular composition of umbilical cord tissue digests and bone marrow aspirate mononuclear cell fraction. (A) The frequencies of different cell populations relative to total viable cells in UC digests (dark bars, n = 5 donors) and BM aspirates (light bars, n = 3 donors). Native MSCs: $CD45^-$, $CD235\alpha^-$ and $CD90^+$. Native ECs: $CD45^-$, $CD235\alpha^-$ and $CD31^+$. **(B)** Representative flow cytometry data showing the gating strategy for the removal of dead/$7AAD^+$ cells (left plot, gate R1) and a distinct native EC population (right plot, gate R2) in UC digest. R1: live cells; R2: positive fraction. **(C)** Distribution of CD146 and CD90 markers on the native EC population of UC tissue digest. Native UC ECs lacked CD90, but 80 ± 16% of them coexpressed CD146 *in vivo*. Dashed lines represent the region used to calculate percentages of marker-positive cells; the data above the lines indicate average proportions of positive cells ± standard deviation (n = 3 donors). *$p < 0.05$.
BM: Bone marrow; EC: Endothelial cell; MSC: Mesenchymal stem cell; UC: Umbilical cord.

to support of angiogenesis (*PGF* and *VEGFA*) were downregulated. Although UC MSCs also upregulated many of the transcripts tested and their response to differentiation stimuli was strong for most transcripts, the expression levels of differentiated BM MSCs were very rarely achieved. These findings potentially suggest that BM MSCs are more predisposed to osteogenesis even in their undifferentiated stage, whereas UC MSCs remain truly 'immature'. Therefore, a combination of our staining (Figure 1B) and molecular (Figure 3 & 4) results support a proposition that UC MSCs might require longer and/or stronger induction to commit to and undergo osteogenesis. The notion of the relative 'immaturity' of UC MSCs additionally explains the higher rate of success in using UC MSCs, compared with BM MSCs, in neuronal applications where a broader differentiation plasticity is needed [55,56].

Apart from MSCs, we were also interested in other cell populations, particularly ECs, in UC tissue digests. We were mindful that in physiological bone remodeling processes and during fracture repair, bone never forms without the presence of vasculature [40]. Whereas BM MSCs could indeed attract and support host vasculature [57], in some cases, including fracture nonunion and large segmental bone defects, an external source of ECs may be required. According to our calculations based on native cell frequencies in BM aspirates, such numbers of ECs could not be achieved from an average single BM donation (it would require approximately 220 ml). A limitation of our work is that only a CD90-based strategy was used to quantify native MSCs; this was owing to its high specificity for MSCs and limited availability of correct CD73 and CD105 antibody conjugates for multiparameter gating required for the analysis of UC tissue digests.

Based on these findings, we propose the feasibility of using UC MSCs/UC ECs mixtures for bone regeneration approaches. ECs are critical for forming the capillary networks connecting to the recipient's circulation, whereas MSCs support and enhance the process of blood vessel formation via chemotaxis and cell-to-cell contact with ECs. Supplementation of UC MSCs with ECs may further accelerate bone formation and maturation [58]. This statement can be further supported by previous data showing the safety of allogeneic UC MSCs in both animal [45,59] and human [60] settings. Importantly, the safety of allogeneic ECs to treat vascular access dysfunction has been recently shown in a multicenter clinical trial [61].

CD146 has been recently proposed as a marker of MSCs in different tissues [38,62] and the fact that it is expressed on ECs [63] has been largely overlooked in the MSC literature. As a part of this study, we showed that CD146 was not only expressed on MSCs, but also on ECs, including native cells. Therefore, CD146-based selection approaches can be potentially used for the isolation of regenerative UC MSC/EC mixtures; such mixtures could enhance tissue vascularization and bone regeneration in the treatment of fracture nonunion or avascular necrosis of the femoral head, where vascular supply to bone is compromised.

Conclusion

Reliable access to UC tissue makes UC MSCs favorable for use as cell therapy. In this study, we showed that although growth characteristics and phenotype maintenance of UC MSCs support their clinical use in principle, their transcriptional and functional profiles in comparison to BM MSCs argue against bone repair applications in which their rapid and direct conversion to osteoblasts is attempted. On the other hand, whole UC tissue digests contain massive amounts of native MSCs and ECs, which without the requirement for culture expansion could be coisolated, banked, injected or used in combination with scaffolds for repairing complex fractures with compromised vascular supply. If native UC MSCs and ECs are proven to be as immunoprivileged as cultured UC MSCs [19,23], these minimally manipulated isolates could provide a novel approach for bone regeneration.

Future perspective

The ultimate goal of MSC-based strategies for bone regeneration is the development of indication- and site-specific approaches that are adaptable to current clinical practices. The disadvantage of using autologous BM as a source of exogenous MSCs for bone regeneration augmentation is its limited supply and dilution with blood when larger volumes are harvested [64]. Complex bone regeneration additionally requires an adequate vascular supply [44]. UC tissue is easily accessible and UC MSCs have been tested previously for their safety in allogeneic settings in humans [65]. The present findings propose a possibility of 'banking' of uncultured cell isolates containing both MSCs and ECs, selected from whole UC tissue digests based on the CD146 expression. Because of the very high number of MSCs and ECs that can be extracted from a single UC tissue donation, the manufacturing and banking costs are likely to be low compared with standard GMP-grade MSC expansion [66] or endothelial

progenitor cell isolation [67]. Furthermore, our findings show good responsiveness of UC MSCs to osteogenic stimulation, indicating that the osteogenic capacity of UC MSCs could be in principle enhanced by using appropriate osteoconductive scaffolds and osteoinductive growth factors such as BMP2 *in vivo* [68]. *In vivo* experiments in animal models of site-specific bone regeneration are therefore warranted to investigate the safety and efficiency of this novel allogeneic cell-based approach of bone regeneration.

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No writing assistance was utilized in the production of this manuscript.

Ethical conduct of research

The authors state that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations. In addition, for investigations involving human subjects, informed consent has been obtained from the participants involved.

Executive summary

Transcriptional profile of umbilical cord & bone marrow mesenchymal stem cells following osteogenic differentiation

- Mesenchymal stem cells (MSCs) from whole umbilical cord (UC) tissue digests respond well to the osteogenic stimulation but display lower *in vitro* differentiation capacity, compared with bone marrow MSCs, due to their lower 'baseline' osteogenesis-related transcriptional activity.

Enumeration of native mesenchymal stem cells & endothelial cells in umbilical cord digests

- UC tissue digests contain large numbers of native MSCs and endothelial cells, which can be enumerated, characterized and copurified based on CD146 expression.
- Specific marker-based strategies should enable a highly selective isolation of minimally manipulated regenerative cell fractions, including MSC/endothelial cell mixtures from the UC tissue, for the development of future therapies for bone regeneration.

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Preliminary Communication

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Regenerative Medicine

Factors influencing yield and neuronal differentiation of mesenchymal stem cells from umbilical cord blood and matrix

Aim: Umbilical cord blood and Wharton's jelly (WJ) are potential sources of mesenchymal stem cells (MSCs). We investigated whether harvesting and donor characteristics affected yield and neuronal differentiation, and compared human umbilical cord blood (hUCB) and WJ-derived MSCs regarding neuronal differentiation and cytokine secretion. **Materials & methods:** MSCs were analyzed by immunoblotting after seven days of differentiation; cytokine protein arrays were used to analyze conditioned medium. **Results:** Birth weight and blood/anticoagulant ratio influenced MSC yield per unit blood volume, but not maternal and gestational age, delivery mode or fetal gender. Expression of the early neuronal differentiation marker nestin was unaffected by these variables. hUCB- and WJ-derived MSC secrete distinct cytokine profiles. **Conclusion:** Cell yield is affected by certain donor characteristics. hUCB- and WJ-derived MSCs may serve distinct therapeutic niches.

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Experiments in animal models of various neurological impairments suggest that the transplantation of mesenchymal stem cells (MSCs) may result in behavioral and morphological improvements. The mechanism of action of such transplantations, however, is not exactly known and subject to debate. Some lines of evidence suggest that there is a functional integration of the transplanted cells into the diseased neural network, in other words, the transplanted cells differentiate into neural-like cells [1,2]. Others observed that improvements were attributable to an indirect mode of action, putatively mediated by factors secreted by the transplanted cells and positively affecting the host tissue [3,4].

Umbilical cord blood has been shown to be a source of multipotent hematopoietic and nonhematopoietic stem cells [5]. Transplantation of such cells into the CNS of animal models of various diseases (e.g., stroke, perinatal hypoxic-ischemic brain injury, spinal cord injury, amyotrophic lateral sclerosis and traumatic brain injury) led to measurable improvements in some cases. However, the mechanisms by which these cells exert their beneficial effects, are not fully understood yet.

In a previous study, the mononuclear fraction of human umbilical cord blood (hUCB) was shown to have the capability to proliferate and to differentiate into neural-like cells in response to different growth factors. Also, the secretion of various cytokines and other factors were modulated in conditioned medium, for example, anti-inflammatory, neuroprotective, angiogenic and chemotactic factors [6]. Specifically, it was demonstrated that the expression of neural marker proteins was induced, while the CD45-expressing lymphocyte population substantially decreased, indicating either transdifferentiation or lineage selection. Furthermore,

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analysis of the medium conditioned by hUCB-derived mononuclear cells after induction with a combination of either EGF and FGF-2 or retinoic acid (RA) and NGF revealed substantial secretion of a number of cytokines, chemokines and growth factors. Many of the secreted factors are renowned for their beneficial effects under inflammatory or brain-damaging conditions, providing anti-inflammatory effects or acting pro-angiogenic. The influence of clinical variables on the cytokine and chemokine secretome and the neuronal differentiation of hUCB-derived MSCs, however, has not been studied to date.

Apart from hUCB, Wharton's jelly (WJ) is another viable source of MSCs [7]. WJ has many advantages compared with hUCB. For example, it is easily available and can be additionally collected in all patients donating hUCB. Moreover, WJ is also available when hUCB cannot be used due to infectious contamination. Lastly, WJ is still available in cases when not enough volume of hUCB has been collected, for example in very early preterm newborns or after umbilical cord milking or delayed cord clamping. However, MSCs from WJ are not identical to those derived from hUCB. For example, MSCs from hUCB and WJ differ in their functional properties. El Demerdash *et al.* found that WJ-derived MSCs could better differentiate into insulin producing cells *in vitro* and better controlled hyperglycemia in diabetic rats *in vivo* [8]. The differential influence of clinical variables on the cytokine and chemokine secretome and the neuronal differentiation of WJ-derived MSCs compared with hUCB-derived MSCs is unknown.

In the present study, therefore, we aimed to assess whether the amount of collected hUCB volume and maternal and fetal clinical characteristics such as birth weight, maternal age, gestational age, delivery mode and fetal gender affect the cell yield of MSCs and their potential of neuronal differentiation. In addition, we compared the secretory potential regarding cytokines and chemokines of MSCs from hUCB and WJ. This study was designed to improve the understanding of the biology and potential risks and benefits of MSCs when used as potential therapeutic agents in the context of various degenerative disorders, for example, nervous system impairments such as stroke, perinatal hypoxic-ischemic brain injury or spinal cord injury. The results of this study may also be used to direct future research in this area.

Materials & methods

Materials

This study was performed with the approval of the responsible institutional review board. Written informed consent for umbilical cord collection was obtained from all patients. Inclusion criteria for umbilical cord collection were delivery at the department after an uneventful singleton pregnancy and written informed consent. Exclusion criteria were fetal malformation, amnion infection syndrome, maternal drug abuse during pregnancy and use of prescription drugs during pregnancy except for iron, iodine and vitamin preparations. Cord blood was obtained by sterile puncture of the umbilical vein immediately after delivery of the fetus and stored according to established procedures [9]. The umbilical cord was kept in Hank's balanced salt solution containing penicillin (100 IU/ml) and streptomycin (100 µg/ml) at 4°C. Both, blood and cord were processed between 2 and 36 h after delivery.

Isolation & culture of hUCB-derived mononuclear cells

Umbilical veins were punctured postpartum and the blood was collected in tubes containing citrate phosphate dextrose (CPD) as anticoagulant. Blood samples were stored for up to 24 h before mononuclear cells were isolated by density gradient centrifugation (d = 1.077 g/ml; Biocoll, Merck-Millipore/Biochrom, Berlin, Germany). The mononuclear cell fraction was collected from the interphase, resuspended in phosphate-buffered saline (PBS) and the number and viability of cells were assessed by trypan blue staining. Further culture and expansion of cells were carried out as described previously [6]. In brief, cells were plated at a density of 1×10^7 cells/ml in Dulbecco's minimal essential medium, supplemented with 2 mM glutamine, 0.001% β-mercaptoethanol, 1× nonessential amino acids and 10% fetal calf serum (standard medium [SM]). After 2 days *in vitro* (DIV), nonadherent cells were transferred to fresh culture dishes and cultured for another 2 days, during which time they became adherent. Subsequently, cells were expanded in proliferation medium, consisting of 50% Dulbecco's minimal essential medium/50% Ham's F12, supplemented with EGF (20 ng/ml) and FGF-2 (20 ng/ml). After a total of 7 DIV, cells were transferred to neural differentiation medium (DM), in which EGF and FGF-2 were substituted by all-trans RA (0.5 µM) and NGF (100 ng/ml), and then cultured for another 7 days.

Isolation & culture of Wharton's jelly-derived cells

Explant cultures were obtained essentially as described by Mitchell *et al.* [10]. In brief, blood vessels were removed from umbilical cords and the remaining tissue was diced into small fragments. The fragments were rinsed in PBS, drained and placed on dry culture dishes. After 10 min, SM was added and cells were allowed to migrate/grow for approximately 2 weeks.

Cells were passaged when they reached 80% confluency. After the initial passage, explant cultures were treated the same as hUCB-derived cells.

Preparation of mononuclear cell-conditioned medium

Conditioned medium was collected from cells after 7 days in DM (i.e., after a total of 14 DIV for hUCB-derived cells and 10 days after the initial passage for matrix-derived cells) and analyzed either immediately or stored at -20°C in bovine serum albumin-coated cryotubes.

Immunocytochemical analysis & immunoblotting

Determination of cell morphology and expression of neural markers was performed as described previously [6]. In brief, for immunocytochemistry, cells grown on coverslips were fixed in 4% paraformaldehyde (in PBS) for 20 min, blocked and incubated with primary antibodies overnight at 4°C, followed by three 5-min wash steps and secondary antibody incubation for 2 h at room temperature. For immunoblotting, total cell lysates were prepared by directly adding sodium dodecyl sulfate (SDS)-sample buffer (Roti-Load 1, Carl Roth, Germany) to cells. In all cases, cells from passage numbers 2 to 4 were used.

Human cytokine protein array & ELISA

Expression levels of secreted factors in the conditioned media were assessed by a commercial cytokine antibody array (RayBiotech Inc., GA, USA) as described previously [6]. Specifically, we investigated the potential of the cells to differentiate, defined as the percentage of cells adopting a neural phenotype and expressing corresponding markers and measured the quantitative and qualitative secretion of cytokines (IL-1α, IL-1β, IL-6, IL-7, IL-8, IL-10, IL-12, IL-13, IL-16), growth factors (ANG, VEGF, BDNF, GDNF, NT-3, NT-4, PDGF-B, EGF, HGF) and chemokines (MCP-1, MCP-4, MIP-1β, MIP-3α, SDF-1, Etx-2, PARC, MIG, GRO).

Statistics

Chi-square test was used for the comparison of frequencies and cross-tabulations, and student's t-test (normally distributed data) or Mann–Whitney U test was used on means. Descriptive statistics (means, standard deviations [SDs]; median and ranges) were calculated. For multivariate logistic analysis, variables were categorized as indicated. We used the statistical software SigmaPlot 12.5 for Windows (Systat Software Inc., CA, USA).

Results

We collected 205 hUCB samples, of which 104 (51%) were derived from male newborns and 101 (49%) were derived from female newborns. Table 1 shows a comparison of clinical variables such as gestational age, delivery mode (vaginal delivery vs cesarean section), mother's age, birth weight of the newborn and the blood/CPD ratio between male and female hUCB samples.

Clinical factors influence the cell yield of MSCs

Figure 1 shows box and dot blots of the MSC cell counts per ml in relation to the newborn's gender, delivery mode, gestational age, mother's age, birth weight of the newborn and the blood/CPD volume ratio, demonstrating that the MSC cell count was significantly affected by the birth weight of the newborn as well as by the blood/anticoagulant (CPD) volume ratio. Specifically, a higher birth weight led to a higher MSC count (in millions per ml blood) (median: 2.86 [interquartile range: 1.82–4.47] vs 2.13 [1.65–2.93]; p = 0.017), whereas a lower blood/CPD ratio led to

Table 1. Donor characteristics.

Variable	Total	Male	Female	p-value
n	205	104 (51)	101 (49)	
Gestational age (days)	273.6 ± 10.0 (273; 235–294)	272.9 ± 10.0 (273; 247–290)	274.4 ± 10.1 (273; 235–294)	0.3
Delivery mode: – Vaginal – Cesarean	 119 (59) 82 (41)	 64 (63) 38 (37)	 55 (56) 44 (44)	0.4
Mother's age (years)	29.3 ± 5.7 (29.3; 15.9–45.0)	29.9 ± 5.6 (30.0; 16.1–44.4)	28.6 ± 5.9 (27.8; 15.9–45.0)	0.1
Birth weight (g)	3320 ± 454 (3330; 1890–5050)	3396 ± 435 (3355; 2250–5050)	3243 ± 461 (3250; 1890–4230)	**0.016**
Blood/CPD ratio	3.12 ± 1.86	3.16 ± 1.82	3.08 ± 1.91	0.8

Data are shown case counts (percentage in parenthesis) or as mean ± standard deviation (median; range).
CPD: Citrate phosphate dextrose.

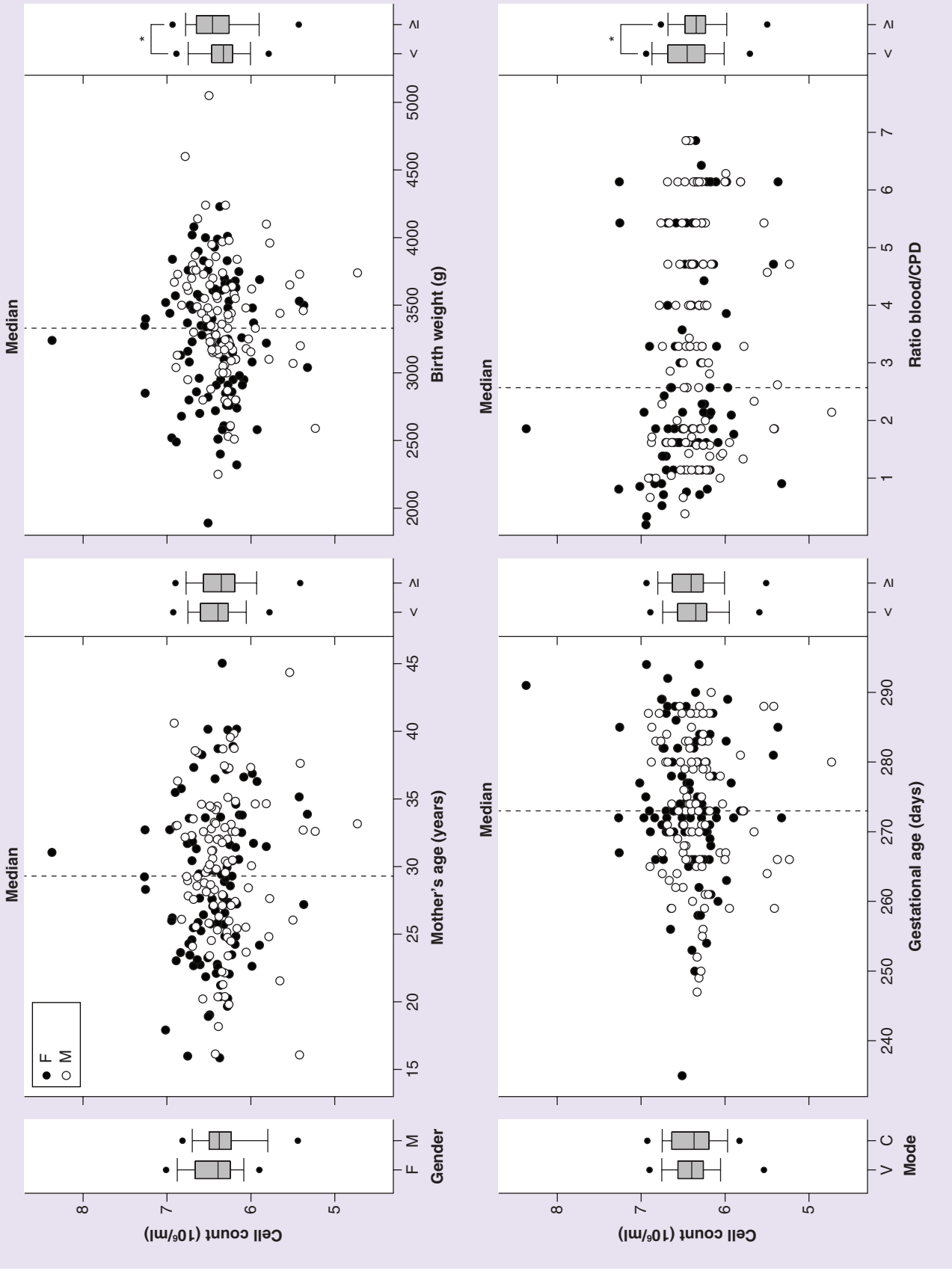
Cell count (10⁶/ml)
Median
Birth weight (g)
Ratio blood/CPD
Mother's age (years)
Gestational age (days)
Gender
Mode
F
M
V
C
<
≥
*

Figure 1. Box and dot plots showing the cell yield per volume umbilical cord blood depending on gender (female; male) and weight of the newborn, mother's age, gestational age, delivery mode (vaginal; cesarean section) and ratio between the volumes of blood and anticoagulant solution (citrate phosphate dextrose solution) (see facing page). Box plots: The boundary of the box closest to zero indicates the 25th percentile, a line within the box marks the median, and the boundary of the box farthest from zero indicates the 75th percentile. Whiskers (error bars) and closed circles above and below the box indicate the 90th/95th and 10th/5th percentiles, respectively. Dot plots: Data from male (open circles) and female (closed circles) newborns are shown. The dotted lines indicated the median value of the x-axis variable. Box plots to the right of dot blots show a comparison of the data < median (<) versus ≥ median (≥). Statistically significant differences are indicated: *p < 0.05.
C: Cesarean section; CPD: Citrate phosphate dextrose; F: Female; M: Male; V: Vaginal.

a higher MSC cell count (2.83 [1.74–4.81] vs 2.21 [1.71–3.00]). The other clinical parameters had no significant impact on the cell count (Table 2). In line with the association of the birth weight and the blood/CPD volume ratio with the MSC yield, there was a significant correlation between these parameters ($p = 0.038$ with a correlation coefficient of 0.146 and $p = 0.002$ and a correlation coefficient of -0.220, respectively). There was no correlation between birth weight and the blood/CPD volume ratio ($p = 0.5$) or the blood volume that was drawn from the umbilical vein ($p = 0.1$), although this was dependent on the delivery mode (33 ml [21–43 ml] for cesarean sections vs 27 ml [15–34 ml] for vaginal births; $p = 0.004$). Multivariate analysis also identified birth weight (odds ratio [OR]: 2.80; 95% CI: 1.51–5.22; $p = 0.001$) and blood/CPD volume ratio (OR: 0.52; CI: 0.29–0.94; $p = 0.031$) as predictors of increased cell yield (Table 3).

Nestin expression in hUCB-derived MSCs & WJ-derived MSCs is not affected by clinical variables

Table 4 shows the ratio of present and absent expression of the early neuronal differentiation marker nestin in MSC cultures derived from hUCB and WJ after seven days in DM. While significantly more hUCB-derived cultures were positive for nestin after the differentiation period compared with WJ-derived cultures (85 vs 31%; $p < 0.001$; Fisher's exact test), nestin expression was not affected by any of the clinical variables investigated, in other words, maternal age, gender of the newborn, delivery mode, gestational age and birth weight in both hUCB- and WJ-derived MSCs. Figure 2 depicts western blot images of bands characteristic of the 240-kD nestin protein in three sample pairs of hUCB-derived and WJ-derived MSCs.

hUCB-derived MSCs & WJ-derived MSCs differ in their secretome

Figure 3 demonstrates the secretory activities of hUCB-derived MSCs and WJ-derived MSCs after 7 days in neuronal DM in direct comparison. hUCB-derived MSCs but not WJ-derived MSCs secrete the two neurotrophic factors NT-3 and NT-4. Cultured hUCB- and WJ-derived MSCs demonstrate partly overlapping but distinct secretion patterns regarding anti-inflammatory cytokines (IL-1, IL-2, IL-6 and IL-8), angiogenic factors (Ang, Leptin and HGF), other cytokines (ENA-78, GRO, G-CSF, TGF, TIMP-1 and TIMP-2) and chemokines of the MCP-family. Specifically, hUCB-derived MSCs exclusively secrete IL-10, VEGF, PDGF BB, MIP-1β, MIP-1δ, eotaxin-2, RANTES, IP-10, NAP-2, OSM, leptin, IGFBP-2, MIF and OPN. These cytokines and chemokines were not secreted by WJ-derived MSCs. By contrast, WJ-derived MSCs exclusively secreted IL-3, IL-15, CCL23, GCP-2 and IFN-γ, which were not secreted by hUCB-derived MSCs. The largest group of cytokines/chemokines was secreted by both cell population, albeit to a variable degree (IL-1α, IL-1β, IL-2, IL-4, IL-5, IL-7, IL-8, IL-12, IL-13, EGF, ANG, HGF, IGF-1, FGF-4, FGF-6, G-CSF, GM-CSF, TGF-β1, MCP-3, MIG, ENA-78, I-309, TARC, BLC, M-CSF, MDC, TNFα, TNFβ, THPO, IGFBP-3, IGFBP-4, OPG, TGF-β3, TIMP-1, TIMP-2). Lastly, we found an overlapping secretory activity for a number of cytokines/chemokines (IL-6, MCP-1, MCP-2, MIP-3α, eotaxin-1, GRO, GROα and TGF-β2).

Discussion

The aim of the present study was to assess whether the amount of collected hUCB volume and clinical characteristics such as birth weight, maternal age, gestational age, delivery mode, fetal gender and blood/CPD ratio affect the cell yield of MSCs and their potential of neuronal differentiation. In addition, we compared the secretory activity of MSCs from hUCB and WJ regarding cytokines and chemokines. We found that the cell yield, but not the neuronal differentiation potential of MSCs from hUCB depends on the weight of the newborn as well as on the volume ratio of collected hUCB and anticoagulant (CPD in this case).

Specifically, a higher birth weight led to a higher cell count, whereas a lower blood/CPD ratio led to a higher cell count. The other clinical parameters had no significant impact on the MSC yield. Of note, fetal gender and the delivery mode did not affect MSC cell count and secretome. Others suggested that cesarean section positively influences MSC cell count and cord blood volume [11]. This is in line with our findings when considering total cell numbers, but in our low-

Table 2. Cell yield per unit volume umbilical cord blood.

Variable	Cell count (10^6/ml)		
	n	Median (IQR)	p-value
Overall	205	2.45 (1.71–3.84)	
Gender:			
– Male	104	2.38 (1.71–3.14)	0.1
– Female	101	2.46 (1.76–4.61)	
Delivery mode:			
– Vaginal	119	2.50 (1.82–3.65)	0.7
– Cesarean	82	2.34 (1.56–4.31)	
Mother's age:			
– <Median (29.3 years)	103	2.46 (1.86–4.00)	0.2
– ≥Median	102	2.25 (1.56–3.69)	
Gestational age:			
– <Median (273 days)	110	2.23 (1.66–3.67)	0.3
– ≥Median	94	2.53 (1.82–4.25)	
Birth weight:			
– <Median (3330 g)	101	2.13 (1.65–2.93)	**0.017**
– ≥Median	100	2.86 (1.82–4.47)	
Blood/CPD ratio:			
– <Median (2.57)	106	2.83 (1.74–4.81)	**0.015**
– ≥Median	99	2.21 (1.71–3.00)	

Bold p-values represent statistical significance. Data missing for delivery mode (n = 4), gestational age (n = 1), and birth weight (n = 4). p-values were calculated using the Mann–Whitney U test.
CPD: Citrate phosphate dextrose; IQR: Interquartile range.

risk obstetric population, the MSC cell yield per unit blood volume was identical in vaginal and cesarean deliveries. The difference in blood volume yield may be due to the collection technique with delayed sampling in vaginal deliveries versus immediate sampling in cesarean sections [12]. In addition, the expression of the neuronal differentiation marker nestin was not affected by any of the clinical variables investigated, also confirming that delivery mode and fetal gender do not affect the biologic potential of MSCs. Thus, the ideal MSC donor has a high birth weight and the blood/CPD ratio should ideally be <2.5. This is particularly relevant when collection devices are used which already contain a fixed volume of CPD. Based on our results, it may be advisable to use a collection device that allows for measuring the collected blood volume and to only then add the appropriate amount of CPD. This, however, may not be a practical approach in all settings due to timing and sterility constraints.

Table 3. Multiple logistics regression analysis.

Independent variables	Odds ratio (95% CI)	p-value
Mother's age:		
– ≤Median (29.3 years) vs >median	0.93 (0.51–1.69)	0.8
Gestational age:		
– ≤Median (273 days) vs >median	0.87 (0.46–1.65)	0.7
Birth weight:		
– ≤Median (3330 g) vs >median	2.80 (1.51–5.22)	**0.001**
Blood/CPD ratio:		
– ≤Median (2.57) vs >median	0.52 (0.29–0.94)	**0.031**
Newborn's gender:		
– Male vs female	0.95 (0.53–1.72)	0.9
Delivery mode:		
– Vaginal vs cesarean section	0.87 (0.47–1.63)	0.7

Bold p-values represent statistical significance.
CPD: Citrate phosphate dextrose.

We also found that hUCB- and WJ-derived MSCs markedly differed in their secretome and neuronal differentiation potential. This is a new finding adding to the literature characterizing the distinct properties of WJ-derived MSCs [13]. For example, Lee *et al.* described that hUCB-derived and WJ-derived stem cells had distinct characteristics including different growth capacity, distinguishable cell surface markers and distinct adipogenic and osteogenic potentials [14]. We confirm these findings showing that WJ-derived MSCs exclusively secreted IL-3, IL-15, CCL23, GCP-2 and IFN-γ, whereas hUCB-derived MSCs, but not WJ-derived MSCs secreted the two neurotrophic factors NT-3 and NT-4 as well as IL-10, VEGF, PDGF BB, MIP-1β, MIP-1δ, eotaxin-2, RANTES, IP-10, NAP-2, OSM, leptin, IGFBP-2, MIF and OPN. The largest group of cytokines/chemokines was secreted by both cell populations, albeit to a variable degree (IL-1α, IL-1β, IL-2, IL-4, IL-5, IL-7, IL-8, IL-12, IL-13, EGF, ANG, HGF, IGF-1, FGF-4, FGF-6, G-CSF, GM-CSF, TGF-β1, MCP-3, MIG, ENA-78, I-309, TARC, BLC, M-CSF, MDC, TNFα, TNFβ, THPO, IGFBP-3, IGFBP-4, OPG, TGF-β3, TIMP-1, TIMP-2). Lastly, we found an overlapping secretory activity for a number of cytokines and chemokines (IL-6, MCP-1, MCP-2, MIP-3α, eotaxin-1, GRO, GROα and TGF-β2).

These findings are of potential interest, because they indicate that hUCB-derived MSCs and WJ-derived MSCs may be used as therapeutic agents in different indications. For example, WJ-derived MSCs may be of specific interest for therapeutic alleviation of hypoxia-induced brain damage, because WJ-derived MSCs produce higher amounts of TGF, HGF and G-CSF than hUCB-derived MSCs. TGF mediates tissue regeneration after ischemic brain damage [15] and HGF is neuroprotective in neurons exposed to hypoxia [16]. G-CSF can mobilize MSCs into periph-

Table 4. Nestin-expression in mesenchymal stem cell cultures derived from umbilical cord blood and Wharton's jelly after 7 days in differentiation medium.

Variable	UCB-derived MSCs				WJ-derived MSCs			
	n	Positive	Negative	p-value	n	Positive	Negative	p-value
Overall	61 (100)	52 (85)	9 (15)		26 (100)	8 (31)	18 (69)	
Gender:								
– Male	31 (51)	26 (84)	5 (16)	1	9 (35)	3 (33)	6 (67)	1
– Female	30 (49)	26 (87)	4 (13)		17 (65)	5 (29)	12 (71)	
Delivery mode:								
– Vaginal	35 (57)	30 (86)	5 (14)	1	11 (42)	2 (18)	9 (82)	0.4
– Cesarean	26 (43)	22 (85)	4 (15)		15 (58)	6 (40)	9 (60)	
Age (years):	29.4 (15.9–38.7); 28.7 ± 5.2				29.0 (19.0–38.3); 29.2 ± 4.8			
– <Median	31 (51)	26 (84)	5 (16)	1	12 (46)	3 (25)	9 (75)	0.4
– ≥Median	30 (49)	26 (87)	4 (13)		14 (54)	5 (36)	9 (64)	
Gestation (days):	274 (254–294); 275.8 ± 9.3				273 (237–294); 274.5 ± 10.2			
– <Median	29 (48)	25 (86)	4 (14)	1	12 (46)	4 (33)	8 (67)	1
– ≥Median	32 (52)	27 (84)	5 (16)		14 (54)	4 (29)	10 (71)	
Birth weight (g):	3350 (2510–4600); 3376 ± 436				3350 (1960–4600); 3347 ± 463			
– <Median	30 (49)	25 (83)	5 (17)	0.7	13 (50)	3 (23)	10 (77)	0.7
– ≥Median	31 (51)	27 (87)	4 (13)		13 (50)	5 (38)	8 (62)	

p-values are from Fisher's exact test. Values are absolute numbers (percentage in parentheses). For age, gestation time and birth weight, median (range); mean ± standard deviation are given.
MSC: Mesenchymal stem cell; UCB: Umbilical cord blood; WJ: Wharton's jelly.

eral blood where they integrate into injured cerebral tissue and transdifferentiated into neural cells and may benefit the repair of trauma [17]. In addition, WJ-derived MSCs secreted higher amounts of several anti-inflammatory cytokines compared with hUCB-derived MSCs, e.g. IL-4, IL-12 and TGF-β1, but lacked IL-10 and IL-13. IL-6 was expressed at high levels by both cell populations. This also underscores the potential value of WJ-derived MSCs for treating brain damage, since IL-6 has been shown to be instrumental for central tissue regeneration after apoplexy [18].

On the other hand, WJ-derived MSCs have specific shortcomings. For example, only hUCB-derived MSC secreted detectable amounts of the major angiogenetic factors VEGF, PDGF and angiogenin. This is important because WJ-derived MSCs may thus not be ideally suited for treating hypoxic tissue damage after myocardial infarction, where, for example, in myoblast sheet transplantation diminished VEGF expression after heat shock treatment was associated with a lower therapeutic effect [19]. Also, angiogenin is an important pro-angiogenic factor in this regard [20], which we could also not detect in WJ-derived MSCs.

Regarding the potential for neuronal differentiation of hUCB- and WJ-derived MSCs, our data indicate that this is much higher for MSCs isolated from hUCB. However, regarding the aspect of neuronal differentiation, our study has a number of limitations. First, we have analyzed both types of cell populations only at a single time point after switching to the same differentiation conditions. Second, we have only looked at nestin as a marker for (early) neuronal differ-

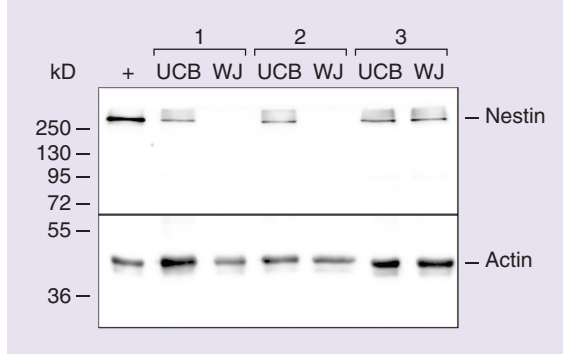

Figure 2. Immunoblot showing the expression of nestin in mesenchymal stem cell populations derived from either Wharton's jelly or umbilical cord blood from three donors. Blots were cut horizontally as indicated by the black line, and stained for actin as loading control. Positions of molecular weight markers are indicated on the left (kD). A rat brain cell lysate served as positive control for nestin (+).
UCB: Umbilical cord blood; WJ: Wharton's jelly.

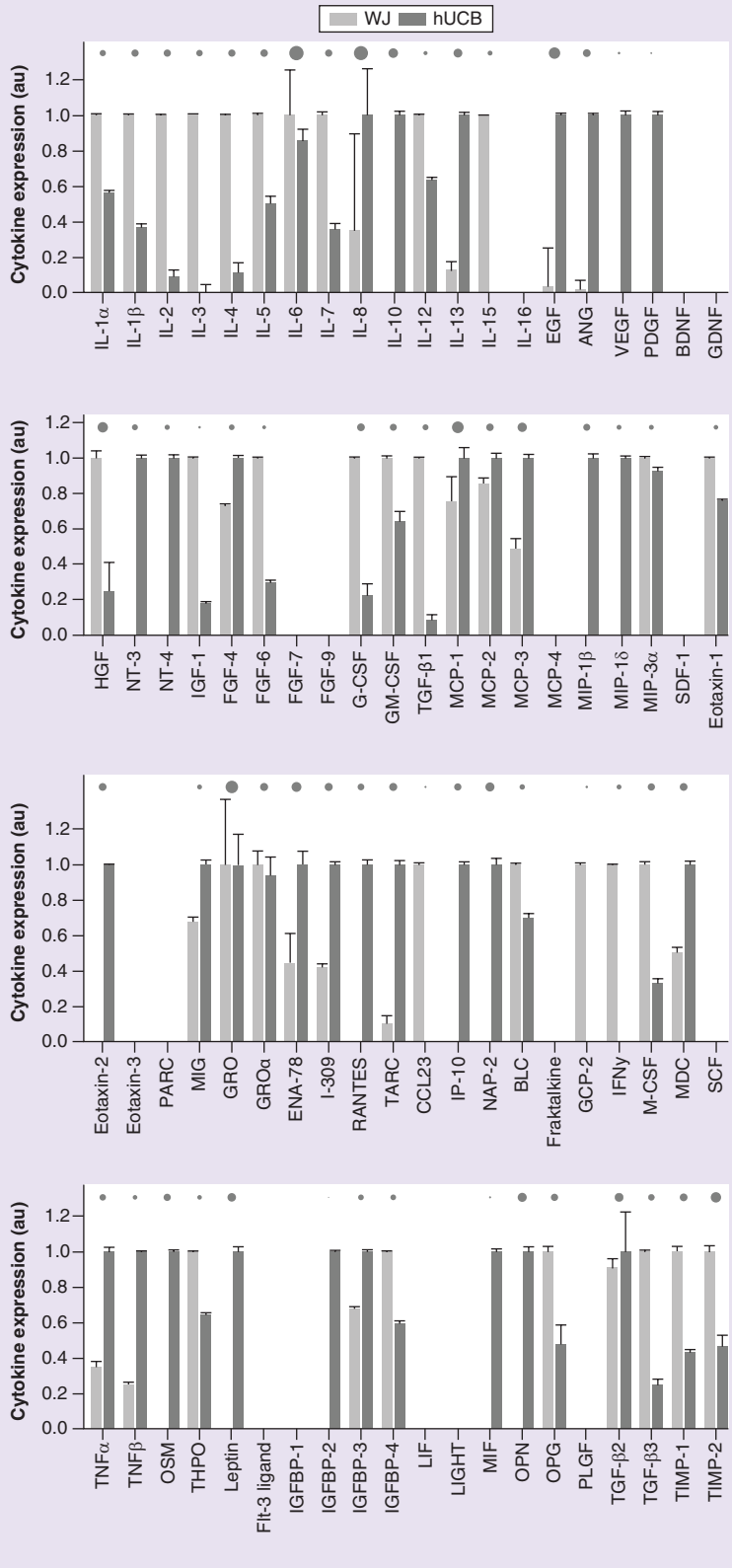
WJ
hUCB
Cytokine expression (au)
0.0
0.2
0.4
0.6
0.8
1.0
1.2
IL-1α
IL-1β
IL-2
IL-3
IL-4
IL-5
IL-6
IL-7
IL-8
IL-10
IL-12
IL-13
IL-15
IL-16
EGF
ANG
VEGF
PDGF
BDNF
GDNF
HGF
NT-3
NT-4
IGF-1
FGF-4
FGF-6
FGF-7
FGF-9
G-CSF
GM-CSF
TGF-β1
MCP-1
MCP-2
MCP-3
MCP-4
MIP-1β
MIP-1δ
MIP-3α
SDF-1
Eotaxin-1
Eotaxin-2
Eotaxin-3
PARC
MIG
GRO
GROα
ENA-78
I-309
RANTES
TARC
CCL23
IP-10
NAP-2
BLC
Fraktalkine
GCP-2
IFNγ
M-CSF
MDC
SCF
TNFα
TNFβ
OSM
THPO
Leptin
Flt-3 ligand
IGFBP-1
IGFBP-2
IGFBP-3
IGFBP-4
LIF
LIGHT
MIF
OPN
OPG
PLGF
TGF-β2
TGF-β3
TIMP-1
TIMP-2

Figure 3. Cytokine expression of Wharton's jelly-derived mesenchymal stem cells (light gray bars) and human umbilical cord blood derived mesenchymal stem cells (dark gray bars) (see facing page). For each cytokine, the expression difference between the two mesenchymal stem cell (MSC) populations is shown relative to the higher expressing population (means and standard error from n = 3 WJ-derived and n = 6 hUCB-derived MSC preparations). The bubbles above the bars give an indication of the relative levels of expression between different cytokines (the area of the bubbles corresponds to the signal value of the higher expressing MSC population, normalized by the assay positive controls).
hUCB: Human umbilical cord blood; WJ: Wharton's jelly.

entiation, and third, we have not quantitated the proportion of cells within a population expressing nestin. In future experiments, these shortcomings should be addressed by looking at differentiation over extended time periods, including additional markers such as the neuronal progenitor markers NCAM and Musashi-1, or the neuronal markers DXC, MAP-2 and TuJ1.

Conclusion

In summary, we found that the cell yield, but not the neuronal differentiation potential of MSCs depends on birth weight and the blood/CPD ratio and that hUCB- and WJ-derived MSCs substantially differ in their secretome and neuronal differentiation potential. hUCB- and WJ-derived MSCs may thus be suitable for different therapeutic purposes.

Financial & competing interests disclosure

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No writing assistance was utilized in the production of this manuscript.

Ethical conduct of research

This study was performed with the approval of the Ethics Committee of the Ruhr-Universität Bochum (registration number 4042-11) and took place in the Department of Obstetrics and Gynecology, Ruhr-Universität Bochum, Marien Hospital Herne. The authors state that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations. In addition, for investigations involving human subjects, informed consent has been obtained from the participants involved.

Executive summary

- Transplantation of mesenchymal stem cells (MSCs) into the CNS have led to measurable improvements in some cases after, for example, stroke or perinatal hypoxic-ischemic brain injury, probably due to secreted factors, including cytokines, chemokines and growth factors.
- Human umbilical cord blood (hUCB) and Wharton's jelly (WJ) are potential sources of MSCs.
- This study aimed to assess whether the amount of collected hUCB volume and maternal and fetal clinical characteristics such as birth weight, maternal age, gestational age, delivery mode and fetal gender affect the cell yield of MSCs and their potential of neuronal differentiation.
- MSCs were analyzed by immunoblotting after 7 days of differentiation; human cytokine protein arrays were used to analyze conditioned medium.
- MSC yield (cell count per volume hUCB) was significantly affected by the birth weight of the newborn as well as by the blood/anticoagulant volume ratio.
- Nestin expression in hUCB-derived MSCs and WJ-derived MSCs was not affected by clinical variables.
- WJ-derived MSCs secreted higher amounts of several anti-inflammatory cytokines such as IL-4, IL-12 and TGF-β1, but lacked IL-10 and IL-13, whereas only hUCB-derived MSCs secreted detectable amounts of the major angiogenetic factors VEGF, PDGF and angiogenin; IL-6 was produced by both cell populations at high levels.
- The different secretomes of hUCB-derived MSCs and WJ-derived MSCs may predestine them as distinct therapeutic agents for certain indications.

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• of interest; •• of considerable interest

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REVIEW

Umbilical cord-derived stem cells for tissue therapy: current and future uses

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Organ and tissue transplants provide a means to correct disease but are limited, mostly owing to the lack of available donor tissue. Tissue matching and speed of procurement are important parameters that must be met for a successful graft, however the lack of available donors leads to expanding waiting lists and suboptimal human leukocyte antigen-matching, often leading to reduced transplant success. The discovery of embryonic stem cells and tissue-specific stem cells has provided hope for many patients. Stem cell-based therapy has provided possible new sources of human leukocyte antigen-matched tissue but, before routine clinical application of stem cells becomes a reality, many obstacles must first be overcome. Focusing on umbilical cord blood cells, we discuss some of the challenges that stem cell therapy faces, including obtaining clinically relevant numbers of stem cells and the ability of stem cells to provide for permanent engraftment of multiple tissue types. We discuss possible solutions to these problems, such as *in vitro* stem cell expansion and the differentiation potential of tissue-specific stem cells.

The goal of treating any disease or disorder is to repair, replace or augment the defective cells, tissues or organs involved. Treatment may be at the biochemical level, involving the administration of pharmaceuticals, or at the organ level, using strategies to remove, replace or repair the tissue. For example, many blood disorders have been treated by the replacement of defective, disease-causing blood cells with hematopoietic stem cells (HSCs) from a healthy donor. This results in long-term treatment facilitated by the transplanted HSCs. Whole-organ transplants, such as those involving the heart, lungs or kidney, can also contribute to long-term correction of defects or injury. In whole-organ transplant, long-term correction is provided not by stem cells but by the survival and slow turnover of the mature cells that comprise the donor organ. Advanced stem cell therapeutics aim to correct tissue and organ defects in a targeted manner by supplying stem cells, which will differentiate into the required cells *in situ*. For example, stem cells delivered to damaged heart muscle may engraft and differentiate into cardiomyocytes. Another option being investigated is the differentiation of stem cells *in vitro* to provide a cell patch to correct the damaged area.

A considerable hurdle that must be overcome before stem cell therapy can be successful is obtaining clinically relevant cell numbers, thus taking this promising therapy from the laboratory bench to the patient. Currently, embryonic stem cells can be grown in sufficient numbers, although their differentiation rate to mature functional cells is inefficient. Tissue-specific stem cells, such as pancreatic or neural stem cells, will differentiate efficiently into the tissue of their origin but accessibility of these stem cells is difficult from living donors.

The paradigm for stem cell therapy is the HSC. Till and McCulloch first reported the existence of such a cell type when they injected syngeneic bone marrow (BM) cells into irradiated mouse recipients and observed the formation of multilineage colonies in the spleen [1,2]. This pioneering work led to the initial hypothesis of a stem cell. Subsequent studies demonstrated that cells found within the $CD34^+$ fraction of the BM have permanent grafting potential [3–5]. These same cells can be isolated from mobilized peripheral blood (PB) and are also found in umbilical cord blood (UCB). Additionally, a separate multipotential cell can also be co-isolated with HSCs from the BM, PB and UCB (Figure 1) [6–9]. Other tissue-origin stem cells, some with reported multidifferentiation potential, have also been described for neural [10], muscle [11], retinal [12], pancreas [13], skin [14] and liver tissues [15]. This review will focus on the clinical potential of stem and progenitor cells derived from UCB.

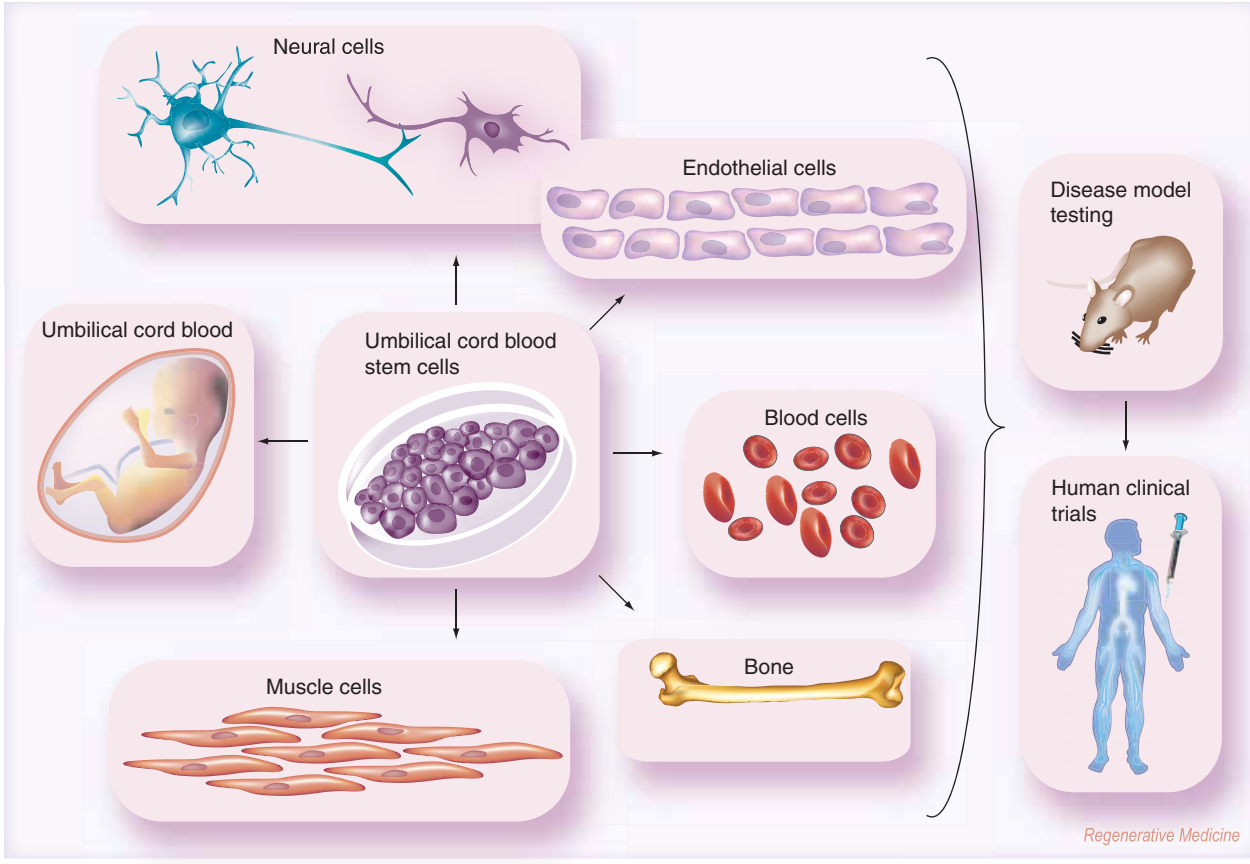
Umbilical cord blood stem cells for hematological disorders

Umbilical cord blood is still considered a novel cell source of HSCs for BM transplantation (BMT), even though the first transplant was carried out

Keywords: cord blood, stem cells, tissue-specific stem cells, umbilical cord-derived

future medicine

Figure 1. Umbilical cord blood is a proven source of hematopoietic stem cells for bone marrow transplantation.



The successful *in vitro* expansion of umbilical cord blood hematopoietic stem cells will provide sufficient cells for routine adult transplants. Umbilical cord blood is also a potential source of multipotential stem cells capable of differentiating into muscle, bone, neural and endothelial cells. Further research, including the development of suitable animal models, is required before umbilical cord blood cell-based therapies is suitable for the clinic.

almost two decades ago. The first successful transplant was performed in France on a patient with Fanconi's anemia using a unit from a related human leukocyte antigen (HLA)-matched donor [16]. Since this initial procedure, thousands of transplants have been carried out worldwide [17]. Treatment of hematological malignancies using donated cells from BM or PB has been successful, but suffers from the lack of suitably matched donors, despite the establishment of databases of potential BM donors. UCB as a source of HSCs has the potential to increase the donor pool.

The speed of procuring samples is a major advantage of UCB. BM registries store information on potential donors that require follow-up contact to obtain cells. The initial identification of a suitable BM donor can take up to 19 days (median), with procurement of cells occurring approximately 30 days later. By contrast, UCB samples are collected at birth with the consent of the mother and can be obtained in a much shorter time period (13.5 days) [18]. Large public cord blood banks that store cryopreserved UCB cells have been established worldwide with linked databases providing easy access to sample information. All of the public UCB storage facilities typically test samples for viability, cell number, $CD34^+$ content, HLA type and infectious diseases prior to cryopreservation, streamlining the process of identifying a suitable UCB unit to transplant.

Factors affecting choice of umbilical cord blood units for transplantation

Currently, the criteria for choosing a suitable UCB unit for transplantation is based on the measured cell content of the unit (in order to provide an adequate cell dose) and the HLA type of the unit.

Although animal studies have demonstrated that a single HSC can result in permanent engraftment of the entire hematopoietic system [19], several studies have demonstrated that increased cell dose is an important determinant of transplant outcomes in humans [17,20,21]. An average UCB unit contains less total nucleated cells and progenitor cells ($CD34^+$) than an average BM aspirate, resulting in lower numbers of infused cells per patient weight in kilograms (cells/kg). For example, studies comparing UCB with BM in both pediatric and adult patients report the median cell doses for UCB transplants are approximately tenfold lower. However, even with lower infused cell doses, these studies demonstrated that, while neutrophil and platelet recovery can be delayed, the incidences of graft-versus-host disease (GVHD), leukemia-free survival, relapse and overall survival was not significantly different for most of the parameters tested [22–25]. Rocha and colleagues report that neutrophil recovery (500 neutrophils/mm^3) was obtained in 26 days for UCB versus 19 days for BM and, by 60 days, 75% of UCB patients had neutrophil engraftment versus 89% for BM recipients. Transplant-related mortality at 2 years was higher in the UCB group (44 vs 38%) although this was not a significant difference. Leukemia-free survival was also similar for both groups (33% for UCBT and 38% for BMT) and no significant difference in mortality rate was observed. Relapse was similar at 23% for both groups [22]. Other studies suggest that UCB cells are more robust engrafters, explaining these positive outcomes [26]. Overall, these results demonstrate the potency of UCB as a graft source and suggest that it may be used as an alternative to BM for HSC transplantation-based therapies.

Clinical studies have considered the effects of cell dose based upon the total number of leukocytes infused, $CD34^+$ cell content and progenitor cell content. Wagner and colleagues analyzed 102 patients and reported that the rate of engraftment is decreased in patients receiving fewer than 1.7×10^5 $CD34^+$ cells/kg body weight (72 vs 93% in patients who received larger doses) [21]. Likewise, it was found that UCB transplants of 3.7×10^8 cells/kg resulted in more rapid engraftment than patients who received only 3.7×10^7 cells/kg (i.e., one log less), although patients receiving the lower cell dose also showed good engraftment [23]. Based on these types of clinical studies, the optimal number of leukocytes required for a successful transplant typically ranges from 20 to 50 million cells/kg [27–29]. The number of colony-forming cells (CFCs) infused has also been correlated to speed of recovery [28]. Methods to increase the size of UCB grafts include the transplantation of two UCB units [30] and *ex vivo* expansion (reviewed below). Some centers have demonstrated the feasibility of using multiple cords for a single transplant; however, procuring two suitably HLA-matched cords may prove difficult in most cases.

The success of UCB transplants may be partially due to the fact that the degree of HLA incompatibility that can be tolerated is greater with UCB than with BM, although different groups have reported conflicting conclusions. Retrospective analysis of UCB transplants for pediatric patients supports the notion that better HLA matching leads to an increase in survival [27]. A comparison of children receiving HLA-matched UCB or BM found that acute GVHD (grade II–IV) was lower for the UCB recipients, with the majority developing grade II. Chronic GVHD was also lower for the UCB group [31]. Similar observations were recorded for adult patients, where UCB mismatched for 1–2 HLAs, matched BM and single HLA-mismatched BM were compared. Acute GVHD was lower in unmatched UCB recipients than in matched BM, but contradictory results arose with regards to the incidence of chronic GVHD [22,24].

By contrast, no correlation between HLA matching and GVHD or survival was reported by Gluckman and colleagues, but HLA matching was shown to be important for engraftment and graft-versus-leukemia effects (GVL) [29]. This discrepancy may be explained by a report from the Institute of Medicine that retrospectively studied adult and pediatric UCB transplantation in the USA. This study found that a matched HLA–UCB unit was better than a unit with one or two mismatches and the severity of the effect could be correlated to dose. At lower cell doses, HLA disparity had a greater effect on survival, suggesting that an increased cell dose may offset the negative effects of HLA mismatching [17]. Similarly, the Eurocord registry analyzed 1000 cases of unrelated cord blood transplants and concluded that, for cord blood transplants, the best indicator of success is not necessarily HLA matching but rather, the number of nucleated cells infused [29]. Despite these findings, HLA matching continues to be a factor in choosing a suitable UCB unit for transplantation [25].

The contribution from donor T cells affects both GVHD and GVL, although the two are not always to be connected [32–34]. The majority of T cells found within the host 3–6 months post transplant are donor derived and express the T-cell marker CD45RO, a marker that is normally associated with T cells emerging from the thymus [35]. After transplant, the T cells secrete interferon-γ and are activated when stimulated with a mitogen, but not by recipient antigens, suggesting the development of tolerance for the recipient and, therefore, reduced levels of GVHD [33]. There is evidence that the adult thymus can educate new T cells that are tolerant to the host tissue [28,36].

While T cells from donor UCB may be less reactive, resulting in reduced GVHD, this may also result in a reduction of the beneficial effects of GVL. The GVL effect is due to cytotoxic T cells of fetal origin present in the UCB that are capable of recognizing the minor histocompatibility antigen HA-1 (a target of GVL), resulting in the lysis of leukemic cells, but is also dependent on the ability of the donor cells to differentiate between donor and recipient [37]. Alteration of the composition of immunity effector cells during transplantation might reduce the severity of GVHD and increase the GVL effect, resulting in increased survival rates for UCB recipients.

Engineering donor effector cells to improve engraftment & survival

Preclinical data suggest that UCB cellular effectors of immunity play an important role in promoting engraftment as well as adoptive immunity through the reduction of GVHD, leukemia relapse and residual disease, or the induction of GVL effects.

Studies in humans have demonstrated that the presence of alloreactive donor natural killer (NK) cells in transplant grafts can reduce incidences of graft rejection, GVHD and leukemia relapse [38]. Using murine models, it was demonstrated that NK cells condition recipients for transplantation by eradicating host lymphohematopoietic cells and obviated GVHD through the targeted elimination of host antigen presenting cells (APCs) [38,39]. Subsequent clinical studies have confirmed the effectiveness of using alloreactive NK cells in stem cell transplantation-based therapies [40]. Regulatory T (Treg) cells that coexpress CD4 and CD25 (IL-2Rα chain) have also been shown to function as potent allogeneic immune modulators [41]. In mice, these cells have been shown to inhibit GVHD while preserving GVL following transplantation [42]. Furthermore, the infusion of *ex vivo* activated and expanded Treg cells prevents GVHD and increases donor cell engraftment [43]. APCs also have importance in modulating GVHD and GVL. Studies have shown that recipient hematopoietic APCs play a primary role in inducing allogeneic T-cell-mediated GVHD [44]. Interestingly, recent data suggest that dendritic cells (DCs), the most potent APCs, derived from macrophage colony-stimulating factor (M-CSF)-containing cultures may provide a method to decrease the incidence of GVHD [45]. The M-CSF-derived DCs (M-DCs) induced a decreased T-cell response and demonstrated tolerogenic potential. It was concluded that M-DCs might have future utility in the suppression of unwanted immune responses *in vivo*, such as GVHD.

Directed adoptive immunotherapy following transplantation has shown applicability. Donor lymphocyte infusion has been used successfully as a pre-emptive therapy for leukemic relapse with the greatest effect observed in patients with chronic myeloid leukemia [32,46]. Recently, a novel application was described that uses engineered donor-derived T cells to eliminate residual disease in patients with B-lineage acute lymphoblastic leukemia. In this study, T cells were genetically modified using a nonviral gene transfer system to express a chimeric immunoreceptor that allowed T cells to recognize the B-cell surface antigen CD19 [47]. T-cells ($CD19^+$R T cells) were capable of cytokine production and $CD19^+$-specific cell lysis *in vitro* and could effectively control $CD19^+$ tumor growth *in vivo*. Infused $CD19^+$R T cells could also be an important consideration in the clinical setting.

It is clear that the role of lymphocytes following stem cell transplantation will become more important as additional preclinical and clinical data are collected. The infusion of specialized immune cells following transplantation may become the standard of care as a method to significantly enhance overall patient outcome.

Secondary complications & infection

The slower neutrophil and platelet recovery rates associated with the use of UCB may increase the risk of secondary complications, especially those due to opportunistic infection [48]. In fact, one study reports that HLA-matched UCB recipients had a 23% mortality rate due to infection, while HLA-matched BM recipients had a 9%

mortality within the first 100 days [31]. The incidence of infection is more serious with UCB transplantation as it can take 12–24 months to reconstitute both the B- and T-cell compartments. T-cell function is slow to recover as the thymus has limited function in adults and is slower to educate T cells. Furthermore, mismatched HLA and the incidence of GVHD contributes to a delay in immune function [49,50]. Opportunistic infections can be an important determinant in overall patient outcome. A Eurocord retrospective study reported that decreased incidences of infection corresponded with increased engraftment rates [51].

Engineering increased stem cell numbers to improve engraftment & survival rates

Using threshold cell dose levels established for BM transplantation, most stored cord blood samples are only acceptable for pediatric patients [52]. Many laboratories have focused on *ex vivo* expansion to increase the number of HSCs in a typical UCB graft. Several studies have achieved expansion, however the cells failed to engraft in mouse models, suggesting that it was progenitor cells and not true stem cells that were propagated. Hematopoietic cells can be classified as mature cells (lin^+) and progenitor cells ($CD34^+/CD38^{+/-}/lin^-$) that have limited renewal capacity, as well as stem cells ($CD34^{+/-}/CD38^-/lin^-$) that have not only a much greater capacity for self-renewal but the ability to confer long-term engraftment [3,53]. Most cell culture regimes result in the expansion of progenitor cells, as measured by phenotypic or *in vitro* functional assays, but limited, if any, long-term repopulation occurs, as demonstrated using mouse BM reconstitution studies [54]. Furthermore, the intracellular interactions between stem cells and progenitor cells makes it difficult to establish whether any stem cell proliferation that does occur is due to the direct effect of exogenous cytokines or indirect effects mediated by nonstem cell populations present in the culture [55].

Ex vivo-expanded UCB cells have been infused into patients along with unmanipulated UCB cells [56,57]. In these studies, there were no obvious toxic effects of the cultured cells but, due to the study design where both cultured and noncultured cells were infused, it was impossible to determine if the expanded cells contributed to engraftment. The recovery times for both neutrophils and platelets as well as graft failure appeared to be consistent with unmanipulated UCB transplants, suggesting no adverse effects of the *ex vivo* expansion. Jaroscak and colleagues evaluated 28 patients receiving *ex vivo*-expanded UCB cells along with unmanipulated cells [57]. Although the majority of patients fared well, they reported that the $CD34^+$ population decreased during culture, suggesting that the expansion of long-term repopulating HSCs did not occur despite an increase in total nucleated cells.

The key barrier to cell expansion is the loss of self-renewing stem cells that occurs during induced cell proliferation. Most hematopoietic cytokines do not have the ability to cause both proliferation and maintenance of the stem cell phenotype. Although the transition from G_1 to S phase can be induced by various growth factors, stem cells, whether neural or hematopoietic, reside mainly in G_0, not G_1 [58]. $CD34^+$ cells induced to enter G_1 using cytokines are less likely to contribute to the repopulation cohort when compared with $CD34^+$ cells treated with cytokines but remaining in G_0 [59,60]. Therefore, cell expansion using exogenous cytokines is a complex process requiring the development of novel culture strategies that allow HSCs to proliferate without triggering differentiation and/or apoptosis.

Within the BM environment, intracellular interactions play an important role in modulating stem cell self-renewal and differentiation [61]. We have shown that similar interactions also affect HSCs in culture. In these studies, we demonstrated that the generation of mature lin^+ cells as well as media composition changes can have significant effects on the *ex vivo* expansion of hematopoietic stem and progenitor cell populations [62]. The integrated use of a cell-selection strategy to remove culture-generated lin^+ cells and media dilution/exchange resulted in increased HSC numbers in comparison with input levels. It was concluded that the mechanism behind the inhibition of HSC expansion in culture was the secretion of negative regulators, either by lin^+ cells or by the indirect stimulation of cells by culture-conditioned media. Suppression of stem cell growth by neighboring cells has also been reported for retinal stem cells [63].

Multipotential stem cells from UCB

It has been well documented that mesenchymal stem or progenitor cells isolated from BM can differentiate into mesoderm-derived cells normally found in the BM (fibroblast, osteoblast, adipocyte and chondrocyte). Adult mesenchymal

cells have also been isolated from adipose (AP) tissue and UCB. Comparative studies have demonstrated similarities and differences between mesenchymal stem cells from different sources [8,64]. One major difference is that mesenchymal cells could be isolated from 100% of the BM or AP samples but only from 29% of the UCB samples. Using the colony-forming units-fibroblast assay, out of 1 million cells plated, 557 colonies formed from AP, 83 for BM but only 0.002 from UCB [8]. The highest senescence occurred for UCB, however, in some samples, the UCB cells demonstrated the highest maximal passage. When compared for their multilineage ability (osteogenic, adipogenic or chondrogenic potential), approximately 70% of the AP or BM samples demonstrated multilineage differentiation, whereas none of the UCB samples had tripotential ability. This is similar to the findings of Wagner and colleagues [65].

Along with the existence of a mesenchymal stem cell, there have been reports of stem cells that seem to be multipotential and capable of differentiating into cells not of their origin. Possible explanations for multipotentiality are: transdifferentiation (nuclear reprogramming), defined here as an alteration in the chromatin that confers an earlier developmental state on a cell similar to events that occur during somatic cell nuclear transfer (SCNT) experiments (the cell de-differentiates and re-differentiates down a different path); the presence of a universal stem cell with wide differentiation potential (embryonic-like); or fusion of cells from two different tissue types.

Nuclear reprogramming has similarities to regeneration and may occur when cells are placed into a different stem cell niche. Although regeneration is not common in mammals, the Murphy Roths Large (MRL) mouse is capable of regeneration in response to wounding [66]. This presents the intriguing possibility that genetic modification may be able to enhance the ability of cells to be reprogrammed. Although it has been argued that reprogramming an adult cell to become a cell type different from its origin is less difficult than trying to create a whole organism, as with 'Dolly' [67], current studies suggest that the frequency of transdifferentiation is extremely low. Murry and colleagues investigated the ability of HSC and BM cells to contribute to cardiac muscle after injury. They demonstrated that purified HSCs were unable to contribute to cardiac muscle of injured hearts as reported by others, but when they followed the fate of BM cells, which include mesenchymal cells, a few cells of BM origin were found in the heart that were positive for cardiac markers (myosin heavy chain). BM cells capable of becoming a cardiomyocyte were extremely rare (one to three cells/heart), which mirrors the inefficiency of obtaining successful nuclear reprogramming by SCNT [68].

A good example in support of a universal stem cell is the BM multipotential adult progenitor cell (MAPC) [6]. MAPCs are not blood cells or HSCs and have a wider differentiation potential than the normal mesenchymal cells found in the BM. MAPCs are capable of differentiating into mesenchymal cell types, as well as cells of ectodermal origin, such as neural cells.

A cell similar to the MAPC has been reported for UCB [7]. Kogler and colleagues reported that 40% of cords used could produce their universal somatic stem cell (USSC). Unlike the MAPCs, the USSCs do not require special growth factor-enriched medium for their establishment. Standard serum-based medium is used in order to induce USSC proliferation. The cells remained undifferentiated until moved to differentiation medium *in vitro* or transplanted *in vivo* into rat or sheep. These cells were capable of differentiation into bone, cartilage, adipocyte, blood, neural and liver cells.

Multipotential cells have been observed in the fetal circulation between 7–12 weeks gestation [69] as well as in the human adult circulation [70]. Although these cells express similar markers to that of BM mesenchymal cells, they have a greater developmental potential (being able to differentiate into muscle, endothelial and neural cells) reminiscent of MAPCs and USSCs.

Embryonic-like stem cells derived from UCB have been reported. These cells express octamer-binding protein (Oct)-4 and Nanog, as well as tumor-rejection antigen (TRA)-1–60, TRA-1–81, stage-specific embryonic antigen (SSEA)-3 and SSEA-4 [71,72]. Although Oct-4 and Nanog are currently recognized as unique to ES cells or the early embryo, there are reports that SSEA-1, SSEA-3 and SSEA-4 are normal components of blood and therefore are not good markers of embryonic phenotype when characterizing blood-derived stem cells [73–75].

It is difficult to draw conclusions on the identity and intrinsic properties of many of these multipotential cells as characterization occurred after *in vitro* culture rather than

immediately upon isolation. The ability of the cell to renew and differentiate into multiple cell types suggests either an embryonic remnant or *in vitro* culture has caused a rare reprogramming event, resulting in a multipotential stem cell. The advantages of finding an ES-like cell from adult tissue, especially UCB or BM, is accessibility; cells could be obtained from living donors easily, thus reducing the long waiting lists for cadaver donors.

Fusion is an important consideration when investigating the multipotential of UCB cells. Fusion has been established as the mechanism for the derivation of hepatocytes from BM mesenchymal cells [76–79] and has been found to occur in other tissues [80]. In fact, some observations of transdifferentiation may be explained as misdiagnosed fusion events. Chromosomal markers are used routinely to determine a fusion event, although this can lead to false-negative results. During the fusion of mouse and human cells, chromosomes are lost through cell division and it is possible that transdifferentiation events are really fusion events where the diagnostic chromosomes have been eliminated. In order to rule out fusion, multiple detection methods must be used in combination, such as detection of multiple chromosomes and human mitochondria as well as tissue-specific markers [81].

Conclusion & future perspective

Before stem cells can be used for clinical therapy, there are specific concerns that that need to be addressed: the accessibility of the stem cells and the ability to obtain adequate numbers of cells to achieve clinically significant improvements. For example, HSCs are easily accessible from BM, PB and UCB and can usually be obtained in clinically significant numbers. By contrast, neural stem cells, which have been well characterized [10] and successful in animal transplant studies, are not easily obtainable. Additionally, these stem cells must also be cultivated 'xeno-free' in order to maintain a clinical grade source of cells.

The interesting but controversial discovery of transdifferentiation offers the possibility of using easily accessible stem cells, such as HSCs, to generate other tissues, including neural or pancreatic. Currently, we do not understand transdifferentiation and its existence is in question. Since the mechanisms underlying transdifferentiation have not been elucidated, this rare event may be explained by fusion or the presence of a rare universal stem cell. Those results initially defined as transdifferentiation may have been derived through the fusion of donor blood cells with recipient organs or the differentiation of a universal stem cell that originally went undetected [81]. The current inability to decipher the events observed *in vitro* or from animal studies inhibits our ability to control these phenomena.

The paucity of unique cell markers and the true determination of what constitutes the functional properties of a cell compound the problem. The distinctiveness of many protein markers is based on their history rather than extensive multicell analysis. Markers first reported as being exclusive to a specific cell type are generally not used on other cells, thus reinforcing their uniqueness. The discovery of a wider profile for 'unique' markers usually comes about serendipitously and is treated with confusion and caution. The field of proteomics could provide us with unique protein profiles of cells that should aid in the identification of stem and progenitor cells. Changes in these profiles during differentiation will also suggest a mechanism while assisting in developing cell culture conditions to enhance the proliferation and differentiation efficiencies of stem and progenitor cells [82–84].

Determining if a stem cell has achieved a tissue-specific functional state is proving difficult. Cell morphology and marker analysis alone do not prove cell function. Although *in vitro* methods do exist for measuring nerve conductance or the myelination of axons by oligodentrocytes or the response of insulin-secreting cells to glucose, the optimal test of cell function is a measurable improvement of the test organism after cell transplantation, although this does not always occur. Mouse models that have been used for decades to test the engraftment potential of HSCs, both human and mouse, provides for an interesting example [1,62]. Despite the long history and extensive use in research, the survival of the mouse is not dependent on the input stem cells. Instead, this animal model is used to identify positive engraftment events, and in some situations the identification and characterization of specific blood subsets. Therefore, assessing success is based on positive engraftment events and not function. In fact, these mice do not support the full development of a functional human immune system. Despite the limitations of this animal model, information

garnered from these studies, along with *in vitro* data, is usually sufficient to commence human clinical trials.

A major challenge of stem cell research will be determining the mechanisms contributing to cell differentiation and tissue repair. Understanding the biological systems and pathways that underlie these complex processes will allow us to modulate stem cell behavior and better harness the therapeutic potential of these cells.

Executive summary

Clinical need for stem cell therapy

- *In vitro* culture of stem cells will provide a continuous supply of human leukocyte antigen-matched cells.
- The ability to control stem cell differentiation *in vitro* will give us the ability to generate specific cell types for targeted tissue therapy.
- It will eventually be possible to generate whole organs or organ systems using stem cells and bioengineering.

Umbilical ord blood transplants for hematological disorders

- Umbilical ord blood transplants for hematological disorders are optimal for pediatric patients due to limited stem cell content.
- Increased success in adults will result from successful hematopoietic stem cell expansion.
- Reduced graft-versus-host disease without loss of the graft-versus-leukemia effect compared with bone marrow hematopoietic stem cells leads to improved survival.
- Engineering grafts containing the requisite cell types will help to improve engraftment outcomes.

Cell engineering

- Self-contained bioreactors that can control growth conditions and cell–cell interactions will improve stem cell proliferation and control differentiation.
- The large-scale production of specific cell types will allow for the production of sufficient cell numbers to treat disease.

Multipotential stem cells

- Isolation and characterization of multipotential stem cells from umbilical cord blood will provide for an easily accessible source of cells that are amenable to harvest and long-term storage.
- Transdifferentiation of tissue-specific stem cells is a rare event but investigation of the underlying mechanisms will lead to favorable conditions and increased efficiency.
- Development of realistic animal models with achievable end points will help to accelerate the transition from laboratory to patient.

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Research Article

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Regenerative Medicine

A comparison of Wharton's jelly and cord blood as a source of mesenchymal stem cells for diabetes cell therapy

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Aim: In this study, we investigated the differences between mesenchymal stem cells (MSCs), isolated from umbilical cord blood (UCB-MSCs) and Wharton's jelly (WJ-MSCs) as sources of diabetes mellitus cell therapy. **Methods:** After isolation, both cell types were induced to differentiate into insulin producing cells, then the differentiated cells were assessed genetically and functionally. UCB-MSCs and WJ-MSCs were transplanted in the tail veins of streptozotocin-induced diabetic rats. Blood glucose levels were monitored post-transplantation. **Results & conclusion:** Wharton's jelly was more homogeneous, can better differentiate into insulin producing cells *in vitro* and better control hyperglycemia in diabetic rats *in vivo*, as compared with UCB. These results indicate that WJ-MSCs represent a potential source of cells in the field of diabetes mellitus cell therapy.

Keywords: cell banking • cell therapy • diabetes mellitus • mesenchymal stem cells • umbilical cord blood • Wharton's jelly

Type 1 diabetes mellitus (T1DM) is an autoimmune disorder in which the body's immune system attacks and destroys pancreatic β cells. While insulin replacement represents the current therapy for T1DM, it hardly controls diabetes, nor does it necessarily prevent the possibility of the disease's devastating long-term complications, which can seriously affect every body organ [1]. Pancreatic or islet transplantation can provide exogenous insulin independence, but is limited by several factors including its intrinsic complications and organ donor's scarcity [2]

The major goal of future diabetes therapy is to promote β-cell regeneration, which could be accomplished by β-cell self-replication or differentiation from progenitor cells with the use of stem cell therapy to overcome autoimmunity and to improve endogenous insulin secretion [2]. Today, significant effort is being made to find alternative means to treat diabetes through stem cell therapy. Several reports have been published concerning the differentiation of many kinds of stem cells into insulin producing cells (IPCs), including embryonic stem cells [3], pancreatic stem cells [4] and mesenchymal stem cells (MSCs) derived from either bone marrow [5] or umbilical cord (UC) [6].

MSCs are uniquely capable of crossing germinative layers borders (i.e., are able to differentiate toward different embryonic lineages including ectoderm-, mesoderm- and endoderm-derived cytotypes) and are viewed as promising cells for regenerative medicine approaches in several diseases [7]. MSCs are obtainable in high numbers via *ex vivo* culture [5]. In addition, other reports evidenced that MSCs possess immune-modulatory activities which should result in a reduction of the immunogenicity of transplanted cells, thus limiting rejection [8]. However, caution is still needed to ensure safe and durable effects of these MSCs *in vivo*.

Interestingly, UC has been proved to be a good source of MSCs either from umbilical cord blood (UCB) or from Wharton's jelly (WJ) – the connective tissue surrounding the umbilical vessels [9,10]. These MSCs offer several advantages over other types of stem cells [7]. For example, UC-MSCs are easily

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isolated compared with embryonic stem cells (ESCs). Moreover, MSCs possess immune-modulatory properties which prevent rejections to occur even after xenotransplantation of postdifferentiated MSCs without immunosuppression [11]. Furthermore, they are obtainable in high numbers and can be differentiated to IPCs. Thus, UC-MSCs represent a promising therapeutic target and a potential source for cell replacement therapy for diabetes mellitus (DM) [12].

MSCs isolated from different tissues using different methods of administration have been used for treating DM in animal models [13,14]. Although these MSCs exhibit several common characteristics, some important differences according to their origin have been shown with regard to their morphology, colony formation abilities, differentiation capacities and therapeutic effects [15,16].

Accordingly, we sought to isolate, propagate and characterize MSCs from two sources of UC, namely UCB-MSCs and WJ-MSCs as noninvasive and readily available sources of stem cells. Furthermore, we compared these two promising types of cells for their pancreatic differentiation potential *in vitro* and their ability to control hyperglycemia in streptozotocin (STZ)-induced diabetic rats *in vivo*. Our results concluded WJ-MSCs as more potential candidate for diabetes cell therapy than UC-MSCs and should be strongly recommended for stem cell banking and DM cell therapy.

Materials & methods

Isolation & culture of UCB-MSCs & WJ-MSCs

The umbilical cords were obtained from Obstetrics/Gynecology Department, Ain Shams after obtaining a signed informed consent from the mother. Fresh human UCB and UC were maintained on ice and processed within 1–4 h post-delivery.

UCB mononuclear cells (UCB-MNCs) were isolated as described previously [17]. Briefly, UCB was collected on sterile 3.2% citrate solution as an anticoagulant. UCB was diluted with phosphate buffer saline (PBS) in the ratio 1:1, then 30 ml of the diluted blood was layered carefully on 10 ml of Ficoll-hypaque (Histopaque 1.077 g/ml density, Sigma-Aldrich, MO, USA) in 50 ml Falcon tubes and then centrifuged at 2000 rpm for 30 min at room temperature. Mononuclear cells were collected from plasma/ficoll interphase and washed twice with PBS. Contaminating erythrocytes were lysed with ammonium chloride lysis solution, and then washed with PBS. Cell density was adjusted to 1–2 × 10^6/ml and seeded in 1 g/l glucose (low glucose)-DMEM (LG-DMEM) supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin. All cell culture media and supplies were purchased from Lonza, Switzerland. Cells were seeded in six-well plates (Coning, USA) till reaching confluency after 14–21 days at 37°C with saturated humidity and 5% CO2 by volume in CO2 incubator (Thermo Scientific, MA, USA). Then, cells were subcultured using 0.05% trypsin-EDTA (Lonza, Switzerand). Fibroblast-like cells started appearing 1 month after isolation. Media for these cells were changed every 2–3 days and sub-cultured once weekly. These fibroblast-like cells were used for subsequent experiments.

As for WJ-MSCs, the UC was collected in sterile PBS and processed within 1–4 h. The UC-WJ was processed till obtaining single cells by explant method as previously described with few modifications [18]. Briefly, the UC was swabbed with 70% alcohol for just few seconds then washed twice by sterile PBS. Afterward, it was cut into smaller pieces (2–5 cm long each). The CB vessels were removed and the remaining tissue was cut into smaller pieces (5–10 mm each). These pieces were placed in six-well plates in LG-DMEM supplied with 10% FBS, 0.02 M L-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin and subsequently incubated in 37°C, 5% CO2 humidified atmosphere (Thermo Scientific, MA, USA). Fresh media was added every other day. Adherent fibroblast-like cells usually appeared in the plate 10–14 days afterward. These cells were subcultured using 0.05% trypsin-EDTA (assigned as passage 1; P1). Media were changed every other day and usually these cells were subcultured every 3–5 days.

Immunophenotyping of different mesenchymal stem cells populations

Both UCB-MSCs and WJ-MSCs at the third passage were trypsinized and washed twice with PBS, then 100,000 cells were incubated at 4°C in dark for 20 min with human monoclonal antibodies labeled with either fluroisothiocyanate (FITC) or phycoerythrin (PE) as follows: CD14 PE, CD34 PE, CD44 PE, CD45 FITC (BD, Pharmingen, CA, USA), CD73 FITC, CD90 FITC, CD105 PE, (Beckman Coulter, Marseille, France). Mouse isotype IgG1 FITC and PE antibodies were employed as controls. Then, the cells were washed and suspended in 500 µl of FACS buffer and analyzed by CYTOMICS FC 500 Flow Cytometer (Beckman Coulter, FL, USA) using CXP Software version 2.2. All immunophenotyping assays were done in Department of Clinical and Chemical Pathology, Faculty of Medicine, Cairo University, Cairo, Egypt.

Adipogenic & osteogenic differentiation

We performed adipogenic and osteogenic differentiation as examples of specific mesenchymal lineage

using human MSC functional identification kit (R&D systems, Inc., MN, USA). This kit contains specially formulated adipogenic media supplement containing hydrocortisone, isobutyl methyl xanthine and indomethacin in 95% ethanol which can be used to effectively differentiate MSCs into adipogenic lineage. Briefly, cells were cultured in a 24-well tissue culture plate at a density of 3.7×10^4/well in α-MEM Basal Medium supplemented with 10% FBS, 100 U/ml penicillin, 100 ug/ml streptomycin and 2 mM L-glutamine (Lonza) and incubated at 37°C in a humidified atmosphere of 5% CO2. When the cells reached 100% confluency, the medium was replaced with adipogenic differentiation medium to induce adipogenesis. After 5–7 days, lipid vacuoles started to appear in the induced cells. The detection of the resultant differentiated cells was done using Oil red staining (Sigma-Aldrich), as described previously [19]. As for osteogenic lineage, 4.2×10^3 cells were seeded in 24-well plate. When these cells reached 50–70% confluency, the medium was replaced with osteogenic medium supplemented with the kit and kept for 21 days with every 3–4 days change of the medium. Differentiation was confirmed by Alizarin red-S staining (Sigma-Aldrich) for the calcium rich extracellular matrix [19].

Pancreatic lineage differentiation

After two to four passages, both UCB-MSCs and WJ-MSCs were induced to differentiate into IPCs using a three step protocol as described previously [20]. Briefly, P2 ~ P4 cells were induced by 5% FBS high-glucose DMEM (4.5 g/l glucose) for 14 days (step I), then 10 mmol/l nicotinamide (Sigma-Aldrich) was added for 7 days (step II), and then finally 10 nmol/l exendin-4 (Sigma-Aldrich) was added for another 7 days (step III).

RNA extraction & Real-time PCR analysis

Both control undifferentiated UCB-MSCs and WJ-MSCs, together with differentiated IPCs (at steps II and III) were collected. RNA was isolated using Trizol Reagent (Life Technologies, CA, USA) according to the manufacturer's instructions. Briefly, 3×10^6 cells were treated by 1 ml Trizol followed by extraction using chloroform and isopropanol. RNA was precipitated by 80% ethanol. cDNA was prepared by Verso™ cDNA synthesis kit (Thermo Scientific, MA, USA) using 0.5 ug RNA. Each quantitative RT-PCR (qRT-PCR) was done using 4 ng cDNA using SYBR Green Master Mix (Applied Biosystems, CA, USA). GAPDH was used as internal control. ΔΔ Ct method was used to calculate relative expression levels. The mRNA expression of various markers was done by qRT-PCR. Forward and reverse primers for target genes are given in (Table 1). All qRT-PCR analyses were done on Step-One plus qRT-PCR (Applied Biosystems).

Functional assessment of differentiated cells by glucose challenge test for insulin release

The maturity of differentiated IPCs was assessed by its ability to secrete insulin in response to high glucose [21]. Briefly, the differentiated cells were washed twice with PBS then incubated for 1 h in Kreb's Ringer bicarbonate (KRB) buffer supplemented with 5.5 mM glucose at 37°C, 5% CO2. Afterward, cells were incubated with either 5.5 mM, 16.7 mM or 25.5 mM glucose in KRB buffer in the same conditions for 2 h, and then the supernatant was collected and frozen at -80°C till time of assay. Insulin release was detected by Accubind® insulin enzyme-linked immunosorbent assay (Monobind Inc., CA, USA) according to the manufacturer's instructions.

Transplantation of UCB-MSCs & WJ-MSCs into streptozotocin-induced diabetic rats

A total of 40 male Sprague–Dawley rats of 4–5 weeks old and 100–150 gm weight were purchased from the Animal Center of the Nile Company for Pharmaceuticals and Chemical Industries, Cairo, Egypt. Animals were housed in normal cages at controlled temperature (24°C) with a 12:12 h light:dark cycle and had free access to water and chow diet over a 5-week

Table 1. Forward and reverse primer sequences used for quantitative real-time PCR.

Gene	Forward primer	Reverse primer
GAPDH	GCCAAAAGGGTCATCATCTC	TGAGTCCTTCCACGATACCA
Nestin	CGTTGGAACAGAGGTTGGA	AGGCTGAGGGACATCTTGAG
Pdx-1	GGAGCCGGAGGAGAACAAG	CTCGGTCAAGTTCAACATGACAG
MafA	CTGGCCATCGAGTACGTCA	CAGAAGCTGGGCGAGGAG
Ngn-3	TCCAAGTGACCCGTGAGAC	AGTGCCAACTCGCTCTTAGG
Nkx2.2	TCTACGACAGCAGCGACAAC	TTGTCATTGTCCGGTGACTC
Isl-1	ATTTCCCTATGTGTTGGTTGCG	CGTTCTTGCTGAAGCCGATG

adaptation period in animal house facility till reaching the ideal weight of STZ injection (200–300 g). Experimental diabetes was induced in these 40 rats (9–10 weeks age, weighing 200–300 gm) by single intraperitoneal injection of 50 mg/kg BW STZ (Sigma-Aldrich) in 0.1 M citrate buffer [22]. Each rat was injected in the right lower part of its abdomen within 30 min of the STZ solution preparation to avoid the degradation of STZ. One week after STZ injection, the rats were food deprived overnight the day prior to blood glucose measurement. The terminal part of rat tail was rubbed by xylene wetted cotton ball to show the tail veins. After xylene dried out, the tail vein was punctured by needle to obtain blood drop. The blood drop was applied on the strip of glucometer (Bionime, Shanghai, China) to measure fasting blood glucose (FBG). Rats having blood glucose level of 200 mg/dl or greater were considered to be diabetic. These recorded FBG levels were used in the subsequent experiment as day 0 (D0) FBG before cells transplantation. In the same day, bodyweights (BW) of the diabetic rats were measured and recorded as D0 BW. Special care was taken to provide enough water and food for diabetic rats.

Of these 40 STZ diabetic-induced rats, 18 rats were selected randomly for stem cells transplantation. These 18 rats were divided randomly into three groups; control group (seven rats), UCB-MSCs group (five rats) and WJ-MSCs group (six rats). Each group was placed in separate cages. On day of injection, each rat received 2×10^6 freshly trypsinized P3 either UCB-MSCs or WJ-MSCs, each according to its corresponding group, suspended in 300 µl plain LG-DMEM through tail vein. Again, the tail was rubbed with xylene and after dried out, cells were transplanted into tail veins using 1 ml 100 unit 27 gauge insulin syringe (BD) on day 7 after STZ induction of diabetes. Same volume of plain LG-DMEM was injected into control group diabetic rats. FBG and BW were monitored every 10 days up to 8 weeks post-transplantation as discussed earlier in this section.

2 months post-transplantation, 2–3 rats of each group were sacrificed by cervical dislocation and dissected for organs collections. Autopsy samples were taken from the pancreas, livers, kidneys and spleens of rats in different groups, along with normal rats. Hematoxylin and eosin (H&E) stained slides were prepared as described before [23]. Briefly, samples were washed with water followed by gradient alcohol for dehydration. Paraffin tissue blocks of these specimens were prepared and sectioned at 4 µm thickness by sledge microtome. Then, obtained tissue sections were collected on glass slides, deparaffinized, stained by H&E stain and examined under light microscope.

Immunohistochemistry

Immunostaining for insulin was done as previously described [24]. Briefly, paraffin sections were deparaffinized and rehydrated by serial changes of xylene, ethanol and distilled water. Antigen retrieval was done by citrate buffer in steamer at 95°C for 30 min. After blocking, slides were incubated with anti-human Insulin (A0564, DAKO, CA, USA) for 1 h followed by incubation with secondary antibody MACH-2 Horse Radish Peroxidase polymer (Biocare Medical, CA, USA). Color was developed using DAB chromogen kit (DAKO, CA, USA) as per manufacturer's instructions and visualized under light microscope.

Statistical analyses

Data are presented as mean ± standard error of mean. Comparisons between the groups were conducted using one-way analysis of variance and Dunnett's *post hoc* test. These statistical analyses were done using windows-based SPSS statistical package (SPSS version 17.0; SPSS, IL, USA). A p-value of less than 0.05 was considered significant.

Results

Both UCB & WJ are sources of mesenchymal stem cells

As for UCB, isolated cells started to adhere to plastic surface in 5–7 days following density gradient isolation. In the beginning, these cells formed an adherent heterogeneous cell population consisting of round and spindle shaped cells (UCB-MNCs; Figure 1A). Initially, these cells proliferate slowly and reach confluency within 3–4 weeks. Upon subculture, this heterogeneous cell population changed to a homogeneous one with flat fibroblast-like shaped cells (UCB-MSCs; Figure 1B).

On the other hand, adherent cells with fibroblast-like morphology could be observed as early as 10–14 days post-plating of the explants of WJ. As shown in Figure 1C & D, these cells were almost homogeneous resembling MSCs morphology, as further proved by immunophenotyping. These cells were designated as WJ-MSCs.

Immunophenotyping of cells isolated from UCB & WJ

Isolated fibroblast-like cells from both UCB and WJ were characterized by flow cytometry for MSCs and hematopoietic-specific cluster of differentiation (CDs) markers. As shown in Figure 2A & B, immunophenotyping revealed that there exists difference in the expression of these markers in both types of cells. Both types were almost negative for CD14 (monocytes), CD34 (hematopoietic stem cells) and CD45 (leukocyte-specific antigen) with more percentage of

cells expressing these markers in the UCB population (CD14 UCB: 4.8% vs WJ: 1.5%; CD34 UCB: 8.6% vs WJ: 2.6%; CD45 UCB: 13.3% vs WJ: 6.3%). On the other hand, both cell types were positive for MSCs markers CD44, 73, 90 and 105. Interestingly, there were difference in expression intensities of these markers between both types of cells (CD44 UCB: 85.1% vs WJ: 83.6%; CD73 UCB: 71.9% vs WJ: 83.4%; CD90 UCB: 74.1% vs WJ 90%; CD105 UCB: 77.9% vs WJ: 82.2%). These results indicate more homogeneous mesenchymal phenotypic population of WJ-MSCs as compared with those of UCB-MSCs.

Adipogenic & osteogenic differentiation of UCB-MSCs & WJ-MSCs

As a functional assay to confirm MSC identity in isolated cells, we examined the differentiation potential of both of these cell populations. Although isolation yields and immunophenotyping profile differ between these two populations, their mesenchymal lineage differentiation capacity either to adipogenic or osteogenic differentiation remained conserved. As Figure 2C–H shows, both UCB-MSCs (Figure 2C–E) and WJ-MSCs (Figure 2F–H) exhibited both adipogenic differentiation potential; detected by oil red staining of lipid droplets in comparison with control undifferentiated cells and osteogenic differentiation potential of the isolated WJ-MSCs; detected by alizarine-red-S staining for calcium rich extracellular matrix as compared with control undifferentiated cells.

In vitro differentiation of UCB-MSCs & WJ-MSCs into IPCs: morphological changes & gene expression analysis

Following exposure to differentiation protocols, both cell types start to lose their fibroblast-like shape and tend to aggregate by the end of nicotinamide (NA) stage. This goes on with exendin-4 stage. Furthermore, cells start to detach and grow as suspension in the culture medium (Figure 3A–D). However, control cells keep their MSCs like morphology throughout the differentiation period.

In order to evaluate the potential of these types of MSCs to differentiate into IPCs, we examined the gene expression of β cells-related genes by qRT-PCR. We examined *Nestin*; a stem cell marker; *Pdx-1*, *MafA, Ngn-3*, *Nkx2.2* and *Isl-1* as β-cell differentiation markers. As shown in Figure 4A, *Nestin* transcript level was decreased in both differentiated UCB-MSCs and WJ-MSCs. It is obvious here that level of *Nestin* transcript in undifferentiated UCB-MSCs was higher as compared with WJ-MSCs.

This decrease in *Nestin* levels upon differentiation was associated with concomitant increase in expression of β-cells genes in both types of cells including *Pdx-1, MafA, Ngn-3, Nkx2.2* and *Isl-1* either in NA stage or at final differentiation stage as shown in Figure 4B & C. Interestingly, all these genes showed a considerable higher fold increase in final differentiated cells when compared with undifferentiated cells in WJ-MSCs than in UCB-MSCs (*Pdx-1*, UCB: 2.5-fold the expression level in undifferentiated control, WJ-MSCs: 14.7-fold; *MafA*, UCB: 2.5-fold, WJ: 3.1-fold; *Ngn-3*, UCBP: 4.9-fold, WJ-MSCs: 6.4-fold; *Nkx2.2*, UCB: 0.27-fold, WJ-MSCs: 7.6-fold; *Isl-1*, UCB: 1.88-fold, WJ-MSCs: 2.8-fold). These findings indicate that although both cells could potentially differentiate down the pancreatic lineage, WJ-MSCs showed increased potentiality toward differentiation into IPCs.

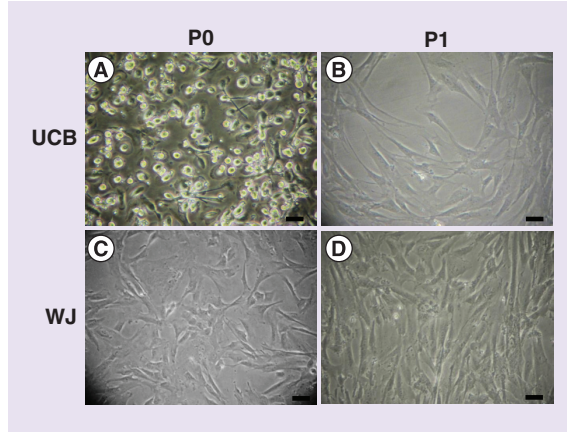

Figure 1. Phase contrast images of umbilical cord blood and Wharton's jelly-isolated cells. (A) Freshly isolated UCB-mononuclear cells showing heterogeneous population, **(B)** UCB-mesenchymal stem cells after first passage (P1) showing homogeneous fibroblast-like cells and **(C)** freshly isolated WJ-mesenchymal stem cells showing homogeneous fibroblast-like cells which continues for P1 as shown in **(D)**.
Magnification: 10×; Scale bar: 100 μm.
UCB: Umbilical cord blood; WJ: Wharton's jelly.

Comparison of glucose-stimulated insulin secretion of IPCs generated from both UCB-MSCs & WJ-MSCs

One important characteristic of β cells is its secretion of insulin in response to glucose [21]. In order to examine the response of differentiated IPCs to glucose stimulation, we incubated differentiated cells with KRB buffer containing a low (5.5 mM) or two high (16.7 and 25 mM) glucose concentrations. ELISA showed a modest secretion of insulin in response to glucose for IPCs isolated from UCB-MSC (LG: 5.62 ± 0.063 μU/ml; high glucose (HG) 16.7mM: 5.77 ± 0.176 μU/ml; HG 25 mM: 5.88 ± 0.290 μU/ml) or WJ-MSCs (LG: 5.21 ± 0.11 μU/ml; HG 16.7mM: 5.93 ± 0.11 μU/ml;

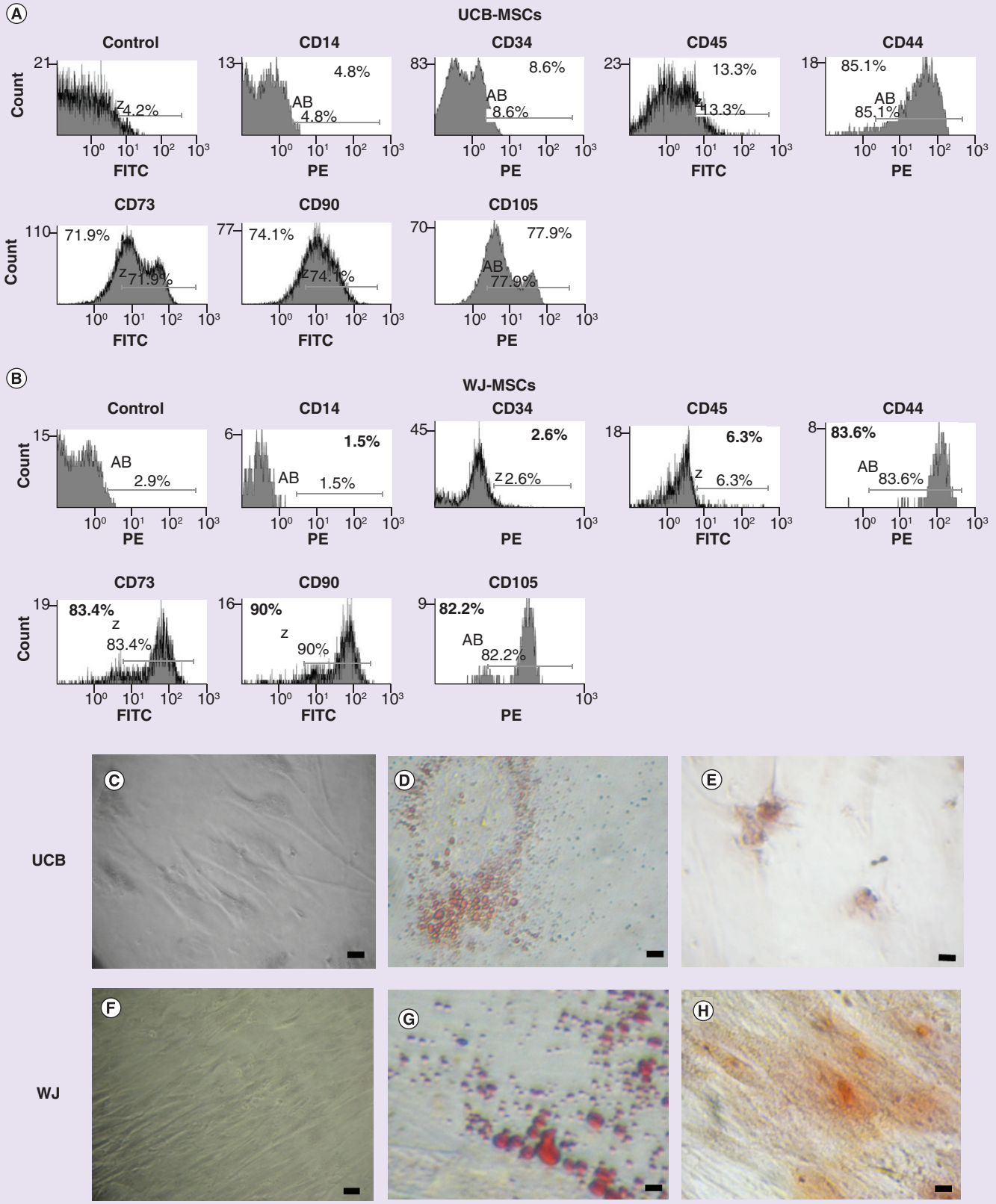
A
UCB-MSCs
Control
CD14
CD34
CD45
CD44
Count
21
4.2%
13
4.8%
AB
4.8%
83
8.6%
AB
8.6%
23
13.3%
13.3%
18
85.1%
AB
85.1%
FITC
PE
CD73
CD90
CD105
110
71.9%
71.9%
77
74.1%
74.1%
70
77.9%
AB
77.9%
B
WJ-MSCs
15
AB
2.9%
6
1.5%
AB
1.5%
45
2.6%
2.6%
18
6.3%
6.3%
8
83.6%
AB
83.6%
19
83.4%
83.4%
16
90%
90%
9
82.2%
AB
82.2%
UCB
WJ
C
D
E
F
G
H

Figure 2. Characterization of mesenchymal stem cells from umbilical cord blood and Wharton's jelly by immunophenotyping and mesenchymal lineage differentiation (see facing page). Immunophenotyping of cells isolated from **(A)** UCB and **(B)** WJ. Cells were labeled with FITC- or PE-conjugated antibodies and examined by flow cytometry. The immunophenotypical profile of both UCB-MSCs and WJ-MSCs showed low expression of CD14, CD34 and CD45 in both types of cells with more cells expressing these markers among UCB. On the other hand, both were positive for CD44, 73, 90 and 105 with more expression intensity for WJ-MSC than UCB-MSC. **(C–H)** Adipogenic and osteogenic differentiation of UCB and WJ-MSCs. **(C)** Uninduced UCB-MSCs as control for lineage differentiation, **(D)** induced UCB-MSCs showing red staining of oil droplets using oil red, characteristic for successful adipogenic differentiation, **(E)** induced UCB-MSCs showing positive alizarin red-S staining for calcium rich extracellular matrix, indicating successful osteogenic differentiation characteristic for MSCs, **(F)** uninduced control WJ-MSCs, **(G)** induced WJ-MSCs showing red staining of oil droplets using oil red, characteristic for successful adipogenic differentiation, **(H)** induced WJ-MSCs showing positive alizarine red-S staining for calcium rich extracellular matrix, indicating successful osteogenic differentiation.
FITC: Fluroisothiocyanate; MSC: Mesenchymal stem cell; PE: Phycoerythrin; UCB: Umbilical cord blood; WJ: Wharton's jelly.

HG 25 mM: 5.79 ± 0.15 μU/ml). However, as shown in Figure 4D, the increase in insulin secretion was slightly higher but significant in WJ-MSCs as compared with UCB-MSCs. Yet, there was no significant variation of insulin secretions between the two high-glucose concentrations.

Transplantation of UCB-MSCs & WJ-MSCs into STZ-induced rats

The diabetes treatment potentials of both types of cells were tested *in vivo* in STZ-induced diabetic rat model. Either UCB-MSCs or WJ-MSCs suspended in plain LG-DMEM media were transplanted into STZ-induced diabetic rats together with another group injected plain LG-DMEM media serving as control group. Fasting blood glucose and BW were recorded every 10 days for 2 months. As shown in Figure 5A, rats transplanted with UCB-MSCs showed decreased FBG starting from day 40 (D40) post-transplantation (UCB-MSCs FBG at D40: 401.2 ± 24.7 mg/dl compared with control D40: 521.0 ± 16.11 mg/dl) and reached its lowest level at D50 (UCB-MSCs FBG at D50: 282.6 ± 29.1 mg/dl compared with control D50: 540 ± 29.80 mg/dl) Unfortunately, these cells could not manage to keep the decreased FBG, which started to elevate to approach control levels at D60 post-transplantation.

On the other hand, the WJ-MSCs transplanted rats showed decreased FBG levels starting at D10 (WJ-MSCs FBG at D10: 209.0 ± 18.5 mg/dl compared with control D10: 378.5 ± 12.85 mg/dl) with sustained decreased FBG levels till D50. However, FBG started to elevate again after D50 where it approached FBG in control group (WJ-MSCs FBG at D50: 439.7 ± 56.2 mg/dl compared with control D50: 540.0 ± 29.80 mg/dl). Then decreased again significantly from control group at D60 (WJ-MSCs FBG at D60: 356.2 ± 45.2 mg/dl compared with control D60: 576.6 ± 8.9 mg/dl). Comparison of the FBG levels between UCB-MSCs group and WJ-MSCs at different time points of the study is shown in Supplementary Figure 1.

Regarding bodyweight, to better examine the effect of cells transplantation on bodyweight, we calculated the bodyweight loss from D0 of transplantation. Figure 5B showed that both UCB-MSC and WJ-MSCs decreased bodyweight loss significantly when compared with control group 1 month post-transplantation and sustained this effect for the next month. These results indicate that, although both UCB-MSCs and WJ-MSCs failed to achieve normoglycemia in STZ-injected rats, they potentially managed to decrease FBG levels in diabetic rats transiently with more efficiency demonstrated by WJ-MSCs in this glucose lowering effect.

UCB-MSCs & WJ-MSCs promoted the recovery of STZ-induced pancreatic damage

In order to examine the *in vivo* effects of MSCs treatment in STZ-induced diabetic rats, we collected autopsy samples of liver, kidney, spleen and pancreas from different treated groups, prepared tissue sections

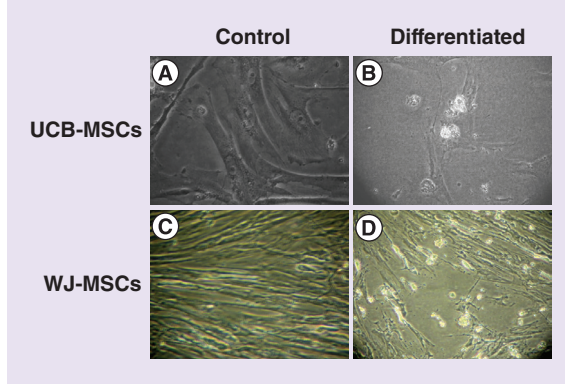

Figure 3. Phase contrast images of differentiated umbilical cord blood mesenchymal stem cells and Wharton's jelly mesenchymal stem cells into insulin producing cells. Upon differentiation, both induced UCB-MSCs **(B)** and induced WJ-MSCs **(D)** aggregate to form clusters in contrast to control UCB-MSCs **(A)** and WJ-MSCs, **(C)** which retain fibroblast-like morphology. Magnification: 10×; Scale bar: 100 μm.
UCB-MSC: Umbilical cord blood mesenchymal stem cell; WJ-MSC: Wharton's jelly mesenchymal stem cell.

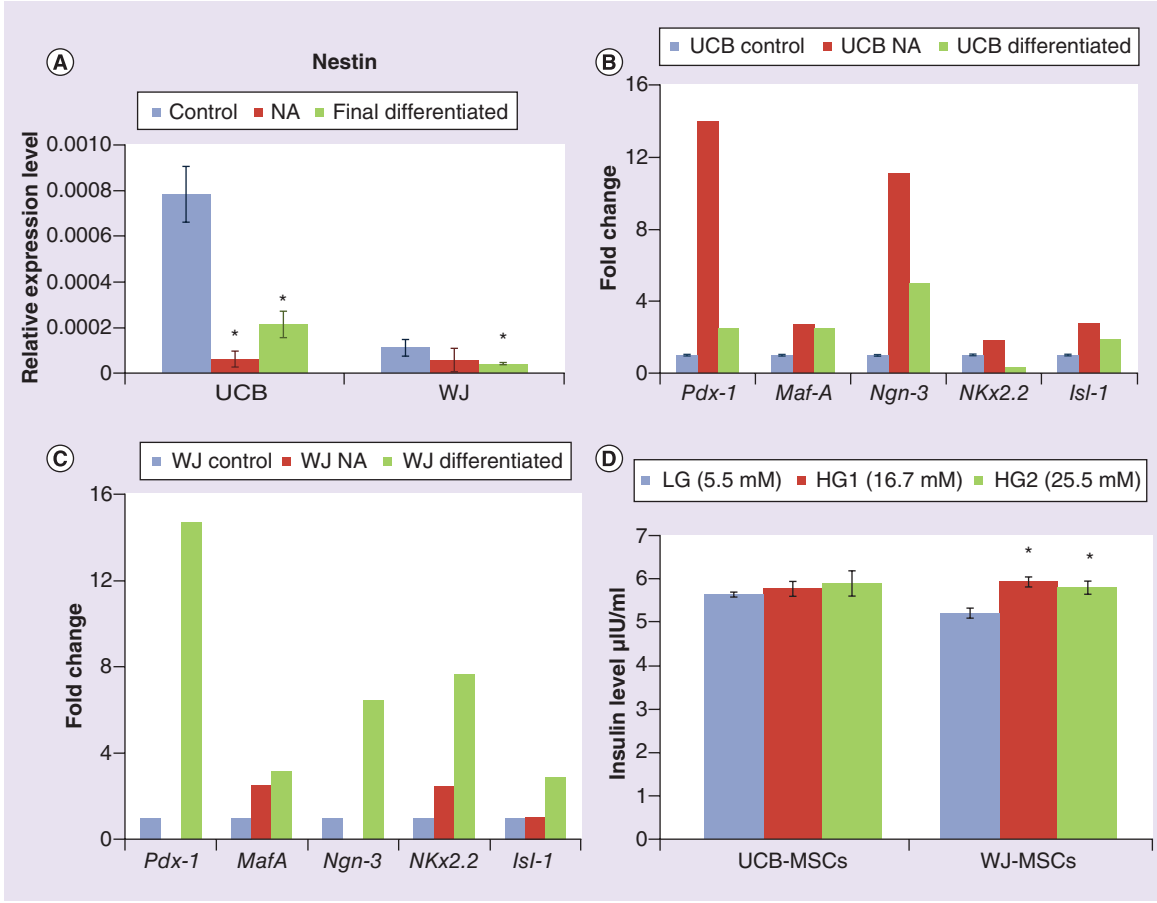

Figure 4. Gene expression of insulin producing cells generated from umbilical cord blood mesenchymal stem cells and Wharton's jelly mesenchymal stem cells. (A) Real-time PCR revealed relative decrease in *Nestin* levels in both UCB and WJ-differentiated cells with increased expression of *Nestin* in noninduced UCB-MSC as compared with WJ-MSC. Fold increase of mRNA relative expression level of β-cells genes in **(B)** UCB-MSC and **(C)** WJ-MSC showed increased expression of *Pdx-1*, *MafA*, *Nkx2.2*, *Ngn-3* and *Isl-1* in both types of cells with more consistent increase associated with insulin producing cells generated from WJ-MSCs. **(D)** *In vitro* GSIS assay of differentiated UCB-MSCs and WJ-MSCs. Insulin release in response to a low (5.5 mM) and two high (16.7 and 25 mM) glucose concentrations of differentiated cell cluster was measured after 2 h incubation.
*Mean is significantly different from control mean at $p < 0.05$.
GSIS: Glucose-stimulated insulin secretion; MSC: Mesenchymal stem cell; NA: Nicotinamide; UCB: Umbilical cord blood; WJ: Wharton's jelly.

and stained them with H&E for routine examination. In addition, we performed immunostaining for human insulin in these pancreata. As shown in Figure 6A & B, the H&E staining of pancreas of the normal control group, showed no histopathological alterations. Meanwhile, it showed some immunostaining for insulin may be due to cross-reactivity of the antibody with other mammalian insulins. As for the rats injected with STZ, expectedly, this group showed dilated pancreatic ducts associated with edema in the periductal tissue. Importantly, the islets of Langerhans were atrophied and showed very little insulin immunostaining (Figure 6C & D).

As for the rats transplanted with MSCs, histopathological examination showed that there was a dramatic improvement in the pancreata of the rats treated with either UCB-MSCs or WJ-MSCs. In contrast to dilated edematous ducts and atrophied islets shown in STZ group, both UCB-MSCs and WJ-MSCs totally restored the normal histological structures of acini and more importantly, islets of Langerhans (Figure 6E & G). This was completely reflected in the positive insulin immunostaining exhibited by the islets in both UCB-MSCs and WJ-MSCs-treated groups. As shown in Figure 6F & H, both cell types showed positive insulin immunostaining in the restored islets. These results obviously point out the ability of these cells to regenerate islets of Langerhans in diabetic rat models.

As for other organ biopsies collected, as shown in Supplementary Figure 2A–C, the H&E staining of liver, kidney and spleen of normal control group, showed no histopathological alterations and normal

histopathological structures of all organs. On the other hand, expectedly, rats injected with STZ (Supplementary Figure 2D–F) showed dilated congested veins associated with inflammatory cells infiltration in between the hepatocytes as well as in the portal area with edema in the later. The kidneys showed degeneration and desquamation in the lining epithelium. In spleen, severe congestion was noticed in the red pulps, while the white one showed lymphoid depletion.

In the rats transplanted with MSCs, Supplementary Figure 2G–I show that the histopathological changes induced by STZ in either liver, including edema and congestion in portal veins, or kidney, including vacuolization in the lining endothelium of the glomerular tufts, were not affected by either transplantation of UCB-MSCs or WJ-MSCs. On the other hand, spleen autopsies showed no histopathological alteration in rats treated with UCB-MSCs (Supplementary Figure 2I). However, spleens of rats transplanted with WJ-MSCs showed lymphoid depletion in the white pulps (Supplementary Figure 2L).

Discussion

In this study, we compared two important banking types of MSCs isolated from UC, namely UCB-MSCs and WJ-MSCs regarding their potentialities toward the generation of IPCs *in vitro* and the control of hyperglycemia in STZ-induced diabetic rats as potential source of DM cell therapy. Results of this study showed that both cell types, UCB-MSCs and WJ-MSCs exhibit typical MSCs characteristics; however, WJ-MSCs were superior to UCB-MSCs in ease of isolation and propagation. In addition, although both cell types failed to attain fully differentiated IPCs *in vitro* or completely ameliorate hyperglycemia *in vivo*, WJ-MSCs exhibited better differentiation potential to IPCs and better sustained control of hyperglycemia in STZ diabetic rats. These results strongly indicate WJ-MSCs can be considered as more potential candidate for DM cell therapy as compared with UCB-MSCs, and should be strongly recommended for stem cell banking.

Stem cells regeneration offers an attractive insulin replacement therapy for those with insulin-dependent DM. Stem cells from pancreas [25], bone marrow [5], UCB [26] have been previously used in research for regeneration therapies for DM. In this context, UC is considered a readily available source of MSCs. In addition, both UCB-MSCs and WJ-MSCs are obtained from tissues that are discarded after delivery, and this nullifies any ethical concerns that might be raised about the use of these cells. Moreover, MSCs have several immune-modulatory properties which enhance their potential for the use in cell therapy of DM [7].

Actually, the International Society for Cellular Therapy suggested three criteria for describing MSCs [27]. The first one is plastic adherence. We succeeded to isolate plastic adherent MSCs-like cells from both types of cells. However, the elapsed time between samples collection and appearance of these MSCs was variable. While it took only about 2 weeks to get the fibroblast-like cells to appear from WJ samples, UCB started with more heterogeneous population, took more than a month to isolate MSC-like cells. Moreover, WJ-MSCs could be expanded more efficiently than UCB-MSCs.

These results supported previous studies on WJ-MSCs in comparison with other MSCs including

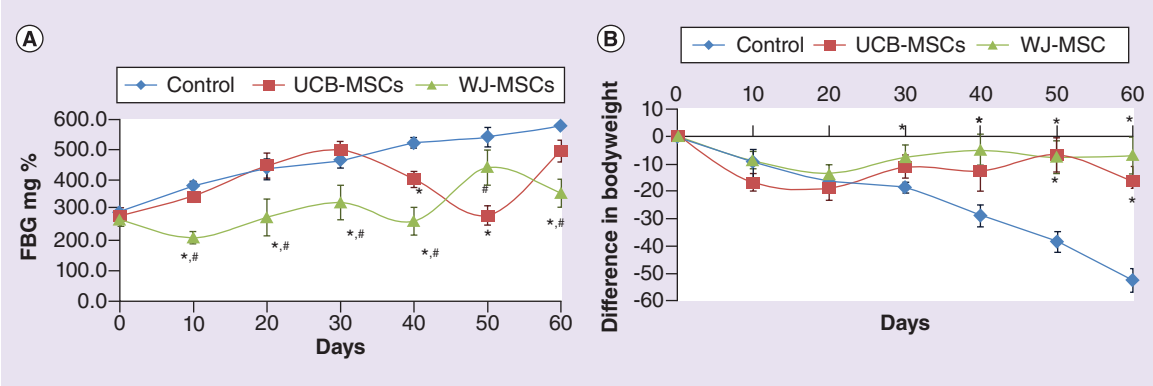

Figure 5. Fasting blood glucose and bodyweight loss in streptozotocin-induced diabetic rats treated with mesenchymal stem cells. (A) Fasting blood glucose and **(B)** bodyweight loss in rats transplanted with UCB-MSCs and WJ-MSCs as compared with control group injected plain Low glucose-Dulbecco's Modified Eagle Medium media. Transplantation of UCB-MSCs and WJ-MSCs decreased FBG and bodyweight loss as compared with control group.
*Mean is significantly different from control mean at $p < 0.05$.
#Mean is significantly different from UCB-MSCs mean at $p < 0.05$.
FBG: Fasting blood glucose; UCB-MSC: Umbilical cord blood mesenchymal stem cell; WJ-MSC: Wharton's jelly mesenchymal stem cell.

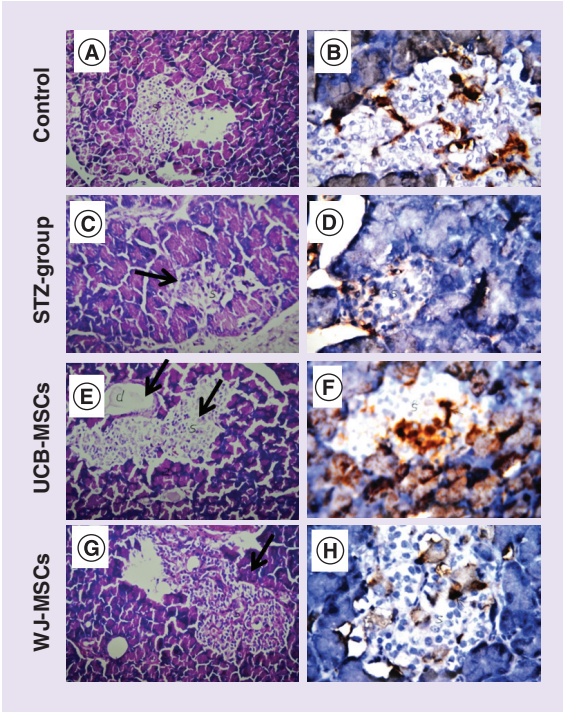

Figure 6. Histology and immunohistochemistry of pancreas in normal, streptozotocin and mesenchymal stem cells-treated diabetic rats. Pancreata in different treated groups were examined using hematoxylin and eosin staining and by insulin immunostaining: **(A & B)** Pancrease of normal rats showing normal histological structure and mild cross-reactivity to insulin; **(C & D)** STZ-treated group showing atrophied islets of Langerhans and weak insulin staining; **(E & F)** STZ diabetic rats treated with UCB-MSCs group showing normal histological structure of pancreas with strong insulin staining; **(G & H)** STZ diabetic rats treated with WJ-MSCs again as in UCB-MSCs showing normal pancreas structure and positive insulin staining.
STZ: Streptozotocin; UCB-MSC: Umbilical cord blood mesenchymal stem cell; WJ-MSC: Wharton's jelly mesenchymal stem cell.

peripheral blood [28] and bone marrow [29]. Taking into consideration that the preference of using cells in clinical applications depends on abundance, frequency and expansion potential of the cells used, our results indicate that WJ-MSCs isolation and expansion properties are more suitable to obtain large-cell number needed for clinical application of cell therapy.

Next criterion proposed by International Society for Cellular Therapy to define human MSCs is the combination of expressing mesenchymal cell surface molecules and lacking hematopoietic ones [27]. Our results showed that MSCs derived from both UCB and WJ fulfilled this criterion. However, WJ-MSCs showed more expression of mesenchymal CDs markers than UCB-MSCs. These results were in accordance with Wu and coworkers, 2009 [29]. This emphasizes that WJ represents a richer source of MSCs than UCB.

In contrast to ease of isolation and immunophenotyping that showed a difference between these two types of cells, both types of cells showed similar multilineage differentiation evidenced by ability of both types to exhibit adipogenic and osteogenic differentiation.

Previous studies showed plausible pancreatic differentiation from embryonic stem cells [30] and bone marrow MSCs [31]. Moreover, several reports have shown the potential of generating IPCs from UCB-MSCs [6,17] and WJ-MSCs [32] or even from other sources of MSCs like adipose tissue [33]. In our study, both types of cells were induced into IPCs after they were cultured in pancreatic differentiation medium containing successive addition of NA and exendin-4 to high-glucose DMEM (4.5 g/l). Basically, differentiation was assessed both genetically and functionally. Interestingly, the more fold increase in β-cells differentiation markers showed by WJ-MSCs as compared with undifferentiated cells upon differentiation into IPCs, which reached almost double that of UCB-MSCs for every gene, suggests a better potentiality of these cells in differentiation into IPCs rather than IPCs generated from UCB-MSCs. Basically, this added more weight to the suitability of WJ-MSCs in cell therapy for diabetes especially in future clinical settings.

However, the formation of mature glucose responsive cells from these sources is a major challenge in the field of diabetes cellular therapy. Several studies have demonstrated that the small amount of insulin secreted by these cells *in vitro* may not be very useful clinically [34]. In addition, several reports assume that stem cells from different origins generate immature IPCs [7].

These previous observations were confirmed in the glucose-stimulated insulin secretion experiment. Although differentiated cells showed increased secretion of insulin upon challenge with high glucose concentration, the response did not significantly change with higher glucose concentrations. This might indicate that resultant cells, although express several β-cell development genes but still did not manage to attain fully functional mature β cells.

Recently, a special attention has been drawn toward the role played by Nestin in differentiation of stem cells into islet-like cells. Nestin is an intermediate filament protein transiently expressed during early development in neuronal cells as well as in embryonic and adult cells [35]. In pancreas, it is considered a marker of pancreatic stem cells and islets progenitor cells [36]. Actually, La Rocca and colleagues, 2009, were among the first groups to show that WJ-MSCs express neuroectodermal marker Nestin [37]. Interestingly, another study showed that MSCs from UC which constitutively express Nestin were ideal candidate source for islet neogenesis and diabetes cell replacement therapy [38]. More-

over, another study demonstrated that knockdown of Nestin by gene silencing in either embryonic stem cells or pancreatic ductal stem cells leads to poor pancreatic differentiation and decreased insulin secretion [39].

However, in our study, *Nestin* transcripts levels decreased upon differentiation in both UCB-MSCs and WJ-MSCs. Interestingly, the transcript level of *Nestin* in UCB-MSCs was higher than that of WJ-MSCs, although WJ-MSCs showed more expression of β-cell genes upon differentiation, that is, showed higher potential toward generating IPCs. In other words, higher *Nestin* expression was associated with cells with lower differentiation potential, which was somewhat different from outcomes from previously mentioned studies. One explanation is that these studies were using either embryonic stem cells or pancreatic stem cells. However, it seems that the role of *Nestin* in pancreatic differentiation of MSCs is quite different and might need further elucidation.

One prominent feature that was shown in both UCB-MSCs and WJ-MSCs is the efficient expression of *MafA* in both types of cells upon differentiation to IPCs. In addition, the expression of *MafA* was more prominent in IPCs generated from WJ-MSCs in comparison with UCB-MSCs. MafA is a transcription factor responsible for insulin synthesis. This indicates that the potential of WJ-MSCs to express insulin gene and secrete insulin in response to glucose will be superior to that of UCB-MSCs. This was confirmed in glucose-stimulated insulin secretion assay, where WJ-MSCs showed slightly higher sensitivity to glucose challenge than UCB-MSCs. Again, we can conclude from these results that WJ-MSCs may represent as a better potential candidate for diabetes cellular therapy than UCB-MSCs.

A number of previous studies and clinical trials have revealed that MSCs are capable of reducing glucose levels in animals or subjects with Type 1 DM [40,41]. Therefore, we considered the transplantation of these two types of MSCs into STZ-induced diabetic rats. Interestingly, the better potential demonstrated by WJ-MSCs *in vitro*, evidenced by higher expression of pancreatic β-cell genes at end stage of differentiation, was completely reflected *in vivo* when these cells were transplanted into STZ-induced diabetic rats. Basically, WJ-MSCs managed to decrease FBG in an earlier and more sustainable fashion as compared with UCB-MSCs. Moreover, the bodyweight loss usually associated with diabetes was greatly improved by both UCB-MSCs and WJ-MSCs transplantation.

These results of our *in vivo* experiments were in accordance with several previous reports. Phuc and coworkers, 2011, showed that IPCs generated from cryopreserved UCB cells were able to just decrease elevation of glucose in STZ-induced diabetic rats as compared with control [17]. In another study, transplantation of IPCs derived from WJ-MSCs into portal vein could significantly decrease blood glucose level in transplanted animals and human C-peptide was detected in livers of transplanted animals [32]. In addition, a recent paper by Si and colleagues, 2012, showed that MSCs treatment ameliorated hyperglycemia in rats with Type 2 DM not only by β-cell restoration but also by improving insulin sensitivity [42]. Interestingly, another recent study by Liu and colleagues, 2014, showed that treatment of Type 2 DM patients with WJ-MSCs could improve metabolic control and β-cell function [43].

It is worth noting here that we used undifferentiated cells in transplantation because undifferentiated cells will provide advantageous use in clinical setting including ease of isolation and expansion with lack of need for manipulation before transplantation. This was the rational after our preference of undifferentiated cells over differentiated ones.

Based on the ability of these MSCs to ameliorate hyperglycemia in diabetic rats, we collected autopsy samples of liver, kidney, spleen and pancreas, which are considered plausible target organs of STZ. Interestingly, H&E staining and insulin immunostaining showed that both UCB-MSCs and WJ-MSCs transplantation ameliorated, at least partially, the damage induced by STZ in the pancreas and produced a similar morphology to normal islets. Based on current knowledge, it was considered that the underlying mechanism of the therapeutic effect of MSCs on hyperglycemia might involve islet regeneration, through direct differentiation into functionally competent β cells [11,44]. Interestingly, a recent study showed that MSCs may promote β-cell regeneration, not through differentiation into β cells, but through cytokine-dependent recruitment of macrophages, which in turn, activate β-cell regeneration [45]. Further studies are warranted to fully elucidate the mechanism by which MSCs can promote β-cell regeneration. Moreover, our results also were consistent with the notion that transplanted MSCs possess tissue repair and cytoprotective properties possibly due to their preferential homing properties to acute-injured tissue [46]. However, in our experiments, MSCs induced tissue repair only in pancreatic lesions with minimal effects on other damaged tissues. Nevertheless, MSCs did not exhibit this effect on any other STZ-induced damaged organ.

It is noteworthy here that some controversial studies have suggested that the limited number of MSC-derived functional β cells *in vivo* and the small

amount of insulin produced by these cells seemed to be inadequate to maintain euglycemia [8,46]. This important concern was completely reflected in our hands both *in vitro* and *in vivo*. Lack of glucose responsiveness in differentiated IPCs *in vitro* together with the inability of MSCs to ameliorate hyperglycemia in diabetic rats, might indicate that although MSCs of either sources exhibit the ability to differentiate into IPCs *in vitro* and restore damaged pancreas *in vivo*, still these differentiated cells did not attain fully functional β-cells phenotype. This might represent a direct challenge in stem cell therapy of diabetes. Future directions and studies should be focusing on how to improve such differentiation if MSCs are to be added to our battle against diabetes.

Actually failure of both cell types to attain fully differentiated IPCs represents a major limitation of this study. Another limitations may include the cross-reactivity of human insulin antibody with rat insulin. This cross-reactivity prevented us from determining if the beneficial effects showed by the MSCs in this study are due to generation human pancreatic cells *in vivo* or regeneration of rat pancreatic β-cells. However, this mechanism warrants further studies for elucidation. Also, we determined the gene expression levels of β-cell markers by detecting the transcript level by qRT-PCR. However, this can be validated by detecting protein levels in further studies.

Another interesting finding revealed in our histopathological study was that WJ-MSCs sustained a lymphoid depletion in spleen indicating an immune-modulatory effect of these MSCs. This was not the case for UCB-MSCs. Several reports have shown the immune-modulatory effect of MSCs and how this might present MSCs as double-edged sword in diabetes treatment [8,47]. Recent studies showed that MSCs could be '*per se*' used in treatment of Type 1 DM [40,48]. This additional benefit gives more weight to WJ-MSCs as a potential candidate in treatment of DM.

Briefly, our findings in this study clearly demonstrate that umbilical cord is a rich source of MSCs, either from UCB or WJ. Interestingly, both represent important cell banking sources for further use by individual during later life. Importantly, our results demonstrate WJ as more rich and readily available source of MSCs when compared with CB. Moreover, WJ-MSCs showed higher differentiation potential toward IPCs *in vitro* and more promising FBG lowering effect *in vivo* in STZ-induced diabetic rats, yet, both types of cells failed to attain fully functional IPCs either *in vitro* or *in vivo*. These findings shed lights on both importance and relative feasibility demonstrated by WJ-MSCs over UCB-MSCs as a potential source of cell therapy for diabetes.

Conclusion

Our results show that human UC represent a rich source of MSCs from either CB or WJ. MSCs isolated from both origins were able to differentiate to pancreatic lineage cells *in vitro* and can alleviate hyperglycemia *in vivo*. Thus, these cells represent a readily available, promising stem cell source for β-cell regeneration. Basically, WJ-MSCs offer advantageous source of cells for diabetes cell therapy when compared with UCB-MSCs. In light of these findings, one would recommend more consideration of WJ-MSCs as source of cell banking for further use later in life. Although the abundance of literature suggests that generation of IPCs from stem cells is feasible, many considerations such as source of cells, induction protocols and mechanisms of differentiation, should be further explored before the application of these cells to clinical treatment of diabetes.

Future perspective

This study is comparing two types of MSCs isolated from UC, namely UCB-MSCs and WJ-MSCs. MSCs are considered strong candidates for use in regenerative medicine and tissue repair. This is attributed in part to their good culture characteristics, which fulfil the need of huge number of cells in clinical applications. In addition, these cells exhibit multipotency, which allow them to generate different organs even from different embryonic lineages. Moreover, these cells may modulate immune responses in host, which may find its way to both allogenic and heterogenic transplantation of these cells. These factors come along side with safety of MSCs, ease of isolation, multiple sources and more importantly nonethical constraints on their use. All of this will make MSCs cells of choice for clinical application of tissue regeneration.

We assume that WJ will be one of the major sources of MSCs that will play an important role in tissue engineering in the near future. Actually, this source of MSCs fulfills all advantageous characteristics needed for the clinical setting and one could easily assume that in the next few years, WJ-MSCs will effectively find their own way to be used in several human diseases.

However, many aspects need to be elucidated before MSCs can be efficiently used for regenerative medicine. First, homing of these cells inside human body and how we can target these cells is an issue of extensive research. Second, methods of culturing of these cells need to be not only reproducible but also fulfil GMP to be applied for clinical use. Third, any unknown effects on the human health must be carefully monitored before these cells can be effectively used in human. Also, these issues will be revealed and

resolved within the next few years and will push the whole world to a new era of tissue regeneration as a new modality for treatment of various diseases.

Supplementary data

To view the supplementary data that accompany this paper please visit the journal website at: www.futuremedicine.com/doi/full/10.2217/rme.15.49

Financial & competing interests disclosure

The authors have no relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript. This includes employment, consultancies, honoraria, stock ownership or options, expert testimony, grants or patents received or pending, or royalties.

No writing assistance was utilized in the production of this manuscript.

Ethical conduct of research

The authors state that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations. In addition, for investigations involving human subjects, informed consent has been obtained from the participants involved.

Executive summary

- Umbilical cord is considered rich source of mesenchymal stem cells (MSCs).
- MSCs can be isolated and expanded from both umbilical cord blood (UCB) and Wharton's jelly (WJ), the connective tissue surrounding the blood vessels of the umbilical cord.

Results

- WJ-MSCs are more abundant, more homogeneous, more easily isolated and expanded than UCB-MSCs, which are more suitable for clinical settings.
- WJ-MSCs can be better differentiated into insulin producing cells than those of UCB evidenced by both genetic and functional assays.
- WJ-MSCs can better control blood glucose levels in streptozotocin-induced diabetic rats when compared with UCB-MSCs.

Conclusion

- WJ-MSCs are better source of MSCs for stem cell banking and regenerative medicine for diabetes mellitus as compared with UCB-MSCs.

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• of interest; •• of considerable interest

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