INTRINSIC PROPERTIES OF MESEMCHYMAL STEM CELLS FROM HUMAN BONE MARROW, UMBILICAL CORD AND UMBILICAL CORD BLOOD

Comparing the different sources of MSC

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Conflict of Interest

The authors declare no potential conflicts of interest.

Abstract

The past decade has witnessed numerous publications on mesenchymal stem cells (MSC), which have great potential in regenerative medicine. MSC from various types of origins exhibit different characteristics, which may relate to the maintenance role of MSC in that specific source. Reports have emerged that among the most widely investigated sources, umbilical cord (UC) or umbilical cord blood (UCB) derived MSC throw advantages over bone marrow (BM) derived MSC due to their close to fetal origin. Here the methodologies used to separate MSC from UC or UCB, and the intrinsic properties, including proliferation capacity, multipotency, cytokine profile, cell surface protein expression and gene expression, between UC, UCB and BM derived MSC, are discussed in details, though may not in a full picture, for the first time.

Key words: Mesenchymal stem cells; Umbilical cord; Umbilical cord blood; MSC heterogeneity;

Introduction

Thirty years ago, Friedenstein first identified fibroblastoid colony forming units (CFU-F) initiated by single cells in mouse bone marrow (BM) [1]. These cells, when they are demonstrated to be self-renewing and multipotent (reviewed in [2]), have been widely termed mesenchymal stem cells (MSC), although questions have been raised about the accuracy of the terminology [3]. They are known to provide the supporting cells for angiogenesis, exhibit a strong immunosuppressive activity which can reduce the incidence of graft-versus-host disease (GvHD) following allogeneic haemopoietic stem cell (HSC) transplantation [4-6] and may also be used for the treatment of autoimmune diseases [7]. Moreover, MSC may be a safer candidate than embryonic stem cells (ESC) for *in vivo* application, as ESC were indicated with karyotypic abnormalities during culture expansion and undergo tumorigenesis *in vivo* which is host-dependent [8, 9] , while MSC are reported with high chromosomal stability and lower tendency to form neoplasm in the recipient [10, 11]. Previous opposite findings about the malignant transformation of MSC were later withdrawn as they were caused by cross contamination of immortal cell lines [12, 13]. Even though MSC turn out to be attractive candidates for regenerative medicine [14-17], and have been universally explored both *in vitro* and *in vivo* in recent years, their nature is yet fully understood.

Bone marrow was the site where MSC were first harvested and the most widely recognized source of MSC, though recent researches have identified several alternative sources with cell characteristics similar to MSC, including adipose tissue (AT) [18, 19], skeletal muscle, dermis (reviewed in [20]), human placenta [21-23], trabecular bone [24], lung [25], umbilical cord (UC) and umbilical cord blood (UCB)[26-34]. It is well recognized that MSC isolated from different origins possess a similar spindle-shaped morphology, and share the core marker profile defined by Dominici [35]. Despite the

morphological similarities, MSC cultures exhibit considerable degree of variabilities in terms of their differentiation potentials and gene expressions, which may in part correlate with cell surface phenotype and with the heterogeneous nature of the cultured cells [2, 36, 37], and these differences should be carefully considered while utilizing MSC for regenerative tissue repair. Umbilical cord and Umbilical cord blood, as extraembryonic tissues that form the link between mother and fetus, represent unique advantages over adult sources, which in conjunction with their abundant availabilities and non-invasive acquisition methodology have attracted a great amount of attention in recent years. In this paper, the phenotype at gene and protein level, and functional variabilities between different sources of MSC were discussed, with a focus on the comparison between BM-MSC, UC-MSC and UCB-MSC.

Current status of isolation of MSC from UCB and UC

Despite that bone marrow represents the main acknowledged source of MSC, the use of BM-MSC is not always appreciable in practice due to the high degree of viral infection and the significant decrease in yield and proliferative capacity with age [38, 39]. Thus, the search for possible alternative MSC sources continues.

The attempt searching for MSC-like cells from human UCB can be traced back to the beginning of this century [30, 40-43]. Other species reported includes equine [44] and ovine [45]. Lee reported successful isolation of MSC from cryopreserved UCB, while Kogler found the efficiency of successful MSC generation from cryopreserved products was scare [46, 47]. It was described that MSC isolated from UCB exhibited two different morphologic phenotypes [43], including flattened fibroblastic cells (majority) and spindle-shaped fibroblastic cells (minority). The spindle-shaped clones might represent superior sources of MSC as it positively expressed CD90 and possessed a greater tendency in

adipogenesis compared to the flattened clones. The success rate of isolating MSC, however, was much lower (7~75%) from UCB [18, 44, 48, 49], while it was 100% from BM, AT and UC [50]. The low success rate of isolation UCB MSC may due to the very low frequency of circulating MSC in cord blood, which was 0.002 ±0.004 per 10^6 initially plated cells whilst the number of CFU-F from BM was $83 \pm 61/10^6$ [18].

The UC contains two arteries and one vein, surrounded by Wharton's jelly (WJ), the connective tissue rich in proteoglycans and hyaluronic acid. Isolation of MSC from umbilical cord has been documented from human [51-55], porcine [56], equine [57] and rat [58]. MSC was identified in UC from several distinct compartments, including WJ [23, 33, 59] and perivascular regions (PV) of both the vein [32] and arteries [60]. Our previous study showed a similar phenotype and multipotency of the MSC from different components of UC, except that MSC from PV were more positive for CD146 expression than those from WJ [61]. Similar to UCB-derived MSC culture, MSC from UC were also revealed to contain two subpopulations [62]: a subpopulation with an average diameter of about 11 μm and a larger sized subpopulation of about 19 μm average diameter. The small-sized subpopulation expressed higher level of CD73 and CD90, and exhibited higher proliferative capacity.

Methodologies of MSC isolation

The isolation of MSC from UCB is relatively simple. A similar protocol as processing bone marrow with Ficoll-Paque density gradient can be adopted. Briefly, the UCB should be collected into a sterile bag containing anti-coagulant, which can be citrate phosphate dextrose [41, 43] or ACD-A buffer [63]. Then it should be diluted with at least an equal amount of PBS or HBSS and separated by density gradient centrifugation on Ficoll Paque. The mononuclear cells should remain in the buffy coat in the

inter phase, which should be collected and washed at least twice before seeded in expansion medium. Crucial parameters of successful isolation include a handling time from collection to isolation of less than 15 hours, a net volume of more than 33 ml, and a MNC count of more than 1 x 10(8) mononuclear cells [41]. The typical density gradient employed was 1.077 g/mL, while recently 1.073 g/mL density gradient was found to have a superior effect in that it more efficiently depleted the CD45(+) fraction and enriched CFU-F to 1.5 fold and the final MSC yield was increased to 1.8 fold after four passages [64]. Besides commonly employed techniques, an osmotic selection method was described with hypotonic conditions (>30min) for isolation of MSC based on their unusual resistance to osmotic lysis, which can enrich CFU-F from UCB for up to 50-fold compared to classic method [31].

Processing UC is quite a different story. A summary of the methodologies of UC-MSC isolation described above was given in Table 1. As UC contains several MSC harboring compartments, various isolation methods have been described. Initially, MSC were harvested from the venous wall of the umbilical cord vein using a protocol well established for isolating human umbilical vein endothelial cells (HUVEC) [53]. Though culture conditions favored the outgrowth of MSC, the digestion of HUVEC at the same time predicts the contamination of HUVEC in the initial culture, and the success rate of separating MSC is relatively low (3 out of 50 samples) [54]. Another method was to mince the whole UC into 1~2 mm fragments and digest in collagenase type II for 30 min and then trypsinize for 30 min [52]. This delivers the advantages of convenience, fast and easy operation, however the cell population generated by this method might contain both endothelial and epithelial cells. A better isolation method was developed in which the mesenchymal tissue was scraped off the WJ after removal of blood vessels, followed by digestion in collagenase for 16 hrs and trypsinization for 30min [33]. Very recently, a two-step protocol was developed by Fong *et al* [50, 65] which is more complicated and

may help to improve the purity of initial population. This included first cutting the cord into small pieces, exposing the inner Wharton's jelly surface to enzymatic solution for 45 min, then separating the Wharton's jelly and homogenizing with a needle. The initial cell population exhibited two types of morphology: epithelioid and short fibroblastic, while only fibroblastic cells remained when passaged. While collagenase is the most useful enzyme to release MSC from the UC tissue, it is also possible to harvest MSC without enzymatic digestion. La Rocca directly put longitudinally sectioned cord fragments with Wharton's jelly exposed into culture medium for 15 days to obtain primary MSC culture based on the "mesenchymal" migratory capability of cells [59], and the isolated UC-MSC were demonstrated to express Oct-1 (octamer-binding transcription factor 1), Oct-4 (octamer-binding transcription factor 4) and Nanog, markers of embryonic stem cells.

The first attempt to separate MSC from perivascular tissue was made by Sarugaser and colleagues, which gained wide recognition by research groups around the world. In this protocol, first the vessels were isolated with surrounding matrix and the two ends were tied to form a loop, which was digested in collagenase for 18-24 hours [32]. In our previous study, the best digestion time was 4 hours, as a digestion longer than 4 hours resulted in the loss of integrity of the vessels [61]. It may not be necessary to seal the two ends of the blood vessel, as attempt has been made with open-ended vessels and no signs of contamination was observed in the isolated cells [28].

Instead of being processed for cell isolation while fresh, it is also reported that UC can be directly cryopreserved in autologous cord plasma after the Wharton's Jelly was extracted and minced [66]. The delaying time between UC collection and processing for cell isolation can be as long as five days with enough cell numbers obtained for transplantation, which greatly facilitates large-scale handling [67].

Some groups used the supplementation of some growth factors, such as EGF, VEGF, or FGF, for the *in*

vitro expansion of UCB-MSC [68, 69] and also for UC-MSC [36, 52, 67, 70], though supplementation of growth factors may not be necessary, as reported for UC-MSC [32, 33, 53, 54] and UCB-MSC [71, 72] in some other papers.

Differences in Self Renewal Capacity

The proliferation and CFU-F forming capacities of MSC from various sources have been studied. Up to date, it was implicated that AT-MSC [18] demonstrated greater proliferation capacity over BM-MSC. This phenomenon could be well explained by another study conducted by Wagner et al [42], where several proliferation related markers, such as Ki-67, cell division cycle associated 8 (CDCA8), and cyclin B2 (CCNB2), were higher expressed in AT-MSC than in BM-MSC. UC-MSC has too been proved to proliferate faster with respect to BM-MSC [52, 55, 73-75]. This may be correlated with a higher CFU-F initial concentration in UC nucleated cells (1:1609 +/- 0.18) than in BM nucleated cells (1:35700 +/- 0.01) [52]. Within the three subpopulations of UC-MSC, namely umbilical cord arterial (UCA)-, venous (UCV)-, and Wharton's Jelly (UCWJ)- derived MSC, UCV cells exhibited a significantly higher frequency of CFU-F than UCWJ and UCA cells did [60]. For UCB-MSC, they were in some reports characterized by very low proliferative activity [76], while in some other cases the proliferation was not restricted by population doublings [41]. When BM-, AT- and UCB- MSC were compared, the CFU-F frequency was highest in AT-MSC, and lowest in UCB-MSC [18].

It should be noted that proliferation capacity and CFU-F ability are not always associated. In Kern's study [18], among MSC from BM, AT and UCB, UCB-MSC had the lowest CFU-F frequency, but could be cultured for the longest period and showed the highest proliferation capacity.

Differences in Multipotency

Despite the fact that MSC from different sources have been illustrated to possess the ability to differentiate down the typical mesodermal lineages, their differentiation patterns differ to some degree. The relevant findings are discussed as below to provide some insight into the distinct differentiation capacities between different sources of MSC, as summarized in Table 2.

Adipogenesis:

Several independent researches indicated that UCB-MSC showed no or a considerably reduced sensitivity to undergo adipogenic differentiation [18, 41, 77]. Induction of adipogenesis in UCB-MSC is difficult, although under certain situation a UCB-MSC strain could produce fat droplets [44, 78], and occasionally with good fortune, could demonstrate higher lipid accumulation compared to BM-MSC [72]. Our previous study [61] has demonstrated that during adipogenesis, the lipid droplets tended to be small and accumulated in the cytoplasm far from the nucleus of UC-MSC lining the cell border resulting in a 'circle' staining appearance, while them tended to be big and gather in the centre of BM-MSC with the morphology looked like a bunch of grape [61]. This is indeed consistent with other group's work as retrieved from the literature [59].

Osteogenesis:

It was reported that compared with BM-MSC, UCB-MSC exhibited better osteogenic capacity. The amounts of Alizarin Red S and alkaline phosphotase activity were 6.9-fold and 2.7-fold greater for UCB-MSC than that for BM-MSC in osteogenic culture, respectively [77]. It was reported that osteogenic differentiation of human UCV-MSC proceeded more rapidly than BM-MSC [75, 79], whereas in our study [61], both UCWJ-MSC and UCV-MSC showed a reduced calcium deposition and no alkaline phosphotase (ALP) activity, which is consistent with the findings of Suzdal'tseva's group

[80]. The gene expression of ALP in UC-MSC was only 8% of that in BM-MSC (unpublished data). Although some researches implicated that AT-MSC lacked the ability to differentiate into osteoblasts [81], in a rat spine fusion model, both collagen sponge containing BM-MSC and AT-MSC transfected with adeno-BMP-2 enhanced new bone formation to a significantly greater extent when compared with the effect of collagen sponge containing BMP-2 only, and the level of the enhancement was comparable [82]. Among UCV-, UCA- and UCWJ- MSC, when analyzed for osteogenic potential, UCWJ cells were the least effective precursors, whereas UCA-derived cells exhibited the best osteogenic potential and developed alkaline phosphatase activity with or without an osteogenic stimulus.[60].

Chondrogenesis:

In chondrogenesis, equine AT-MSC pellets had consistently less and slower GAG expression than BM-MSC pellets, and demonstrated no evidence of collagen II staining, suggesting a reduced chondrogenic potential [83]. UCB-MSC also had limited chondrogenic capacity compared to BM-MSC [72]. Conversely, UC-MSC has been confirmed to express more collagens and glycosaminoglycans than BM-MSC did after 6 weeks differentiation in a polyglycolic acid (PGA) scaffold, however the composition of the collagens UC-MSC produced was mainly collagen type I, whereas collagen type II staining was more intense in differenatiated BM-MSC, leading to the hypothesis that UC-MSC may be a desirable option for fibrocartilage tissue engineering [84].

Untypical lineage differentiation:

Until now, only a few papers have described comparative studies on untypical differentiation of UCB- or UC-derived MSC as opposed to BM-MSC. UC-MSC may have higher endothelial differentiation potential than BM-MSC [74] in that they had higher expression of the endothelial-specific markers

(Flk-1, von Willebrand factor, and vascular endothelial (VE)-cadherin) in endothelial differentiation [73]. Moreover, *in vitro* angiogenesis assay implied that UC-MSC formed higher total length, diameter, and area of tubules than differentiated BM-derived counterparts. In pancreatic differentiation study [73], after treatment with the inductive stimuli, UC-MSC had higher expression of pancreatic-specific markers (PDX-1 and C-peptide) than BM-MSC, intimating their stronger pancreatic differentiation potential. In neuronal induction, UC-MSC also had a higher percentage of neuron specific enolase-positive cells than had BM-MSC [52]. However, UC-MSC or UCB-MSC may not be a good source for stem cell therapy in treating myocardial infarction, as, our previous study [61] has illustrated that after 5-Azacytidine-treatment for cardiomyocyte differentiation, human UC or UCB MSC lack the expression of cardiomyocyte markers (tropomyosin, connexin 43 and Nkx2 5), while a small proportion of BM-MSC did (0.07%).

Differences in Cytokine Profile

Cytokines are small signaling molecules involved in intercellular communication. They serve as molecular messengers in order to regulate the body's response to disease and infection [85]. The cytokine secretion profile of BM-MSC has been confirmed by microarray analysis [86] to be composed of interleukin (IL) -6, IL-8, monocyte chemotactic protein (MCP) -1, Chemokine (C-C motif) ligand 5 (CCL5), growth regulated oncogene-α (GRO-α), Interferon (IFN) -Y, IL-1α, transforming growth factor (TGF) -ß, angiogenin, oncostatin M, macrophage inflammatory protein (MIP)-1ß, IL-2, IL-4, IL-10, IL-12, and IL-13. The expression of granulocyte macrophage colony-stimulating factor (GM-CSF) is debatable. In Potian's study [86], GM-CSF was found to be expressed in BM-MSC, but in another study [52], neither granulocyte colony-stimulating factor (G-CSF) nor GM-CSF was

detected at mRNA level in BM-MSC. Instead, UC-MSC expressed GM-CSF and weakly expressed G-CSF [52], while UCB-MSC did not express G-CSF under serum-free conditions [48].

Parallel comparative analysis of cytokine expression profile of BM-MSC, UC-MSC and UCB-MSC at protein level and mRNA level were retrieved from three publications [65, 66, 87], and statistically re-analyzed in Figure 1. Though some data were not consistent among the three papers, it is noticed that all confirmed IL-6 and IL-8 were upregulated in UC-MSC. In UCB-MSC, the expression of IL-6 was lower, and IL-8 was lower or similar, compared to BM-MSC, as agreed by another report [88]. IL-12(A) and IL-15 expression was similar [66] or higher [65, 87] in UC-MSC in contrast to BM-MSC. IL12(A) is associated with the induction of apoptosis, indicating UC-derived MSC might have better anticancer properties. Though UC-MSC were indicated with higher VEGFR2 expression which enabled a higher response to endothelial induction [65], in Yoo's work [87], VEGF protein expression was much lower in UC-MSC. This result is also supported by Friedman's work [66]. However the changes in protein expression may differs from the gene expression of VEGF, as a high VEGF mRNA expression on UC-MSC was reported in Fong's [65] and Lu's [52] work. From another aspect, VEGFR and VEGF expression may not be related during endothelial differentiation, as Alamino's work [89] showed that during endothelial differentiation, UC-MSC showed upregulated VEGFR1 but reduced VEGF expression.

Differences in Cell Surface Protein Expression

With view to the commonly used panel of MSC markers, our study [61] highlighted a difference in the relative expression of CD146 and CD106 among different sources of MSC. UCB-MSC had higher CD146 expression as compared to BM-MSC or UC-MSC. BM-MSC and CB-MSC were weakly

positive for CD106, while UCV-MSC and UCWJ-MSC were negative, which were consistent with the findings of other groups [52, 61]. Similarly, AT-MSC also showed lower level of CD106 expression compared to BMSC [18, 42, 90].

For the expression of other cell surface molecules, back to 2003, a primitive comparative analysis on a series of surface molecules (including CD3, CD4, CD11c, CD13, CD14, CD15, CD16, CD19, CD29, CD31, CD33, CD34, CD38, CD44, CD45, CD49d, CD54, CD56, CD58, CD62P, CD90, CD104, CD105, CD106, CD144, CD166, SH3 and Stro-1) expressed on human AT-MSC and BM-MSC by flow cytometry, was initiated [91]. Most of the expressions were comparable within the two populations of MSC. Differentially, AT-MSC expressed CD49d (alpha-4 integrin), lacked the expression of CD106 (VCAM-1), highly expressed CD54 (ICAM-1) and lowly expressed CD34, while BM-MSC were CD49d negative, CD106 positive, dimly expressed CD54 and CD34 negative [91]. A higher expression of CD49d was also reported by others in AT-MSC [90] [42] and in UCB-MSC [40]. All these differences detected were within a range of adhesion molecules with known function in homing and mobilization of hematopoietic stem cells. In comparison with BM-MSC, UC-MSC showed down regulation of HLA-ABC [52, 66] and high regulation of HLA-G [59]. A summary of the major difference in surface protein expression of BMSC, UC-MSC, UCB-MSC and AT-MSC was given in Table 3.

Differences in Gene Expression

The first comparative study performed on gene expression of different sources of MSC was initiated in 2004. As expected, MSC from different sources shared a large cohort of gene expression, especially when compared to other types of cells such as HUVEC [92] or HS68 fibroblasts [42], revealing their

close nature in spite of some minor differences.

When BM-MSC and UC-MSC were put into contrast, a set of 45 gene expressions, most of which related to antimicrobial activity and osteogenesis, were at least 10-fold higher expressed in BM-MSC. Conversely, 38 transcripts related to matrix remodeling and angiogenesis were abundantly expressed in UC-MSC while they were absent or rare in BM-MSC [92]. These sources-related characteristics implicated that BM-MSC would be more committed to osteogenesis, whereas UC-MSC would be more committed to angiogenesis [81].

When BM-MSC and UCB-MSC were compared, several genes involved in mesodermal differentiation were noticed [42]. Mesoderm-specific transcript homolog (MEST) was highly expressed in BM-MSC, whereas CB-MSC showed the highest expressions of BMP antagonist 1 (CKTSF1B1 or gremlin 1) and connective tissue growth factor (CTGF). It is also reported that peroxisome proliferation activated receptor gamma (PPARG), which correlated with adipogenic differentiation capacities, and hepatocyte growth factor (HGF) and stromal-derived factor 1 (SDF1/CXCL12), were upregulated in UCB-MSC [72]. Un-induced BM-MSC had higher expression of markers for chondrogenic differentiation (aggrecan 1 (AGC1), collagen X (COL10A1), and cartilage oligomeric protein (COMP)), while UCB-MSC showed upregulated expression of genes associated with embryonic development and organogenesis, including insulin-like growth factor-binding protein 5 (IGFBP5), frizzled-7 (FZD7), rhomboid like protein 2 (RHBDL2), fibulin-1 (FBLN1) and secreted frizzled-related protein 1 (SFRP1) [72].

More recently, meta-analysis of DNA microarray data on UCWJ-MSC, UCB-MSC and BM-MSC pooled from different studies [65] revealed that UCWJ-MSC grouped closest to UCB-MSC and this was perhaps because of their close origin. UCWJ-MSC expressed a highest range of stem cell markers

and lineage markers compared to UCB-MSC and BM-MSC. In contrast to BM-MSC, UCWJ-MSC and UCB-MSC expressed higher number of upregulated oncogenes, and UCWJ-MSC also had more upregulated tumour suppressor gene expression than UCB-MSC and BM-MSC did. Importantly, UC-MSC were found to express some embryonic stem cells markers including Oct-4 [59] and Nanog [79].

As a conclusion, differences in gene expression of MSC from various sources, overall, can be classified into two catalogs. The first catalog consists of genes related to differentiation or proliferation, the other is related to the maintenance of the source where MSC were isolated and possibly reflects the function of MSC within that origin.

Discussion

Bone marrow is the first and most frequently practiced location where MSC can be harvested. Isolation of MSC from BM is facilitated by standard operation procedure and high success rate. However the clinical inconvenience of the invasion procedure and the limited amount of BM available for *in vitro* cell expansion has become a great restriction on its application and leads to numerous searches with inspiration to explore for alternative sources, and to date MSC has been identified from a number of locations. Despite these encouraging discoveries, the nature of MSC from the alternative source is distinctive with respect to their proliferation and differentiation capacities, cytokine profiles, protein and gene expression. This may be to some extent due to the lack of a specific MSC marker, but also the existence of a hierarchy of differentially MSC commitment may play a role [93]. Furthermore, the age of MSC too matters. Although sparse, evidences have been provided that fetal or close to fetal sources contained MSC with relatively undifferentiated phenotype with respect to MSC isolated from adult

sources, which may be due to their longer telomeres [94]. As reported, among fetal BM-MSC, UC-MSC, AT-MSC and adult BM-MSC, only fetal BM-MSC and UC-MSC were positive for the embryonic pluripotency markers Oct-4 and Nanog [79]. Furthermore, MSC from UC and UCB, may be better tolerated following allogeneic transplantation as they are isolated from perinatal tissues [95]. As noncontroversial, inexhaustible sources, and can be harvested noninvasively at low cost, especially with their relatively primitive status, UC- and UCB- MSC are both attractive origins and offer high potential for future application.

As discussed in this paper, since the phenotypes of MSC from different origins are versatile, MSC from a specific source may be more efficient for a particular therapeutic target and should be taken into consideration when choosing MSC source. For example, from the differences in their differentiation potentials, UC-MSC might perform better in blood vessel establishment and cartilage repair, while BM-MSC and UCB-MSC might contribute better to bone repair. UCB- and AT- MSC shall not be considered for chondrogenesis, and UCB-MSC may also not be a good candidate for adipogenesis. UC-MSC has better tumor suppressing properties which makes it a better candidate for anticancer treatment.

To date, the majority of clinical trials are performed with BM-MSC, but as more *in vitro* investigations are going on with other types of MSC, UC-MSC and UCB-MSC start to receive attention in clinical applications. A search for clinical trials using MSC in the public clinical trial database http://clinicaltrials.gov results in 126 records of BM-MSC, 43 records of UC-MSC, and 26 records of UCB-MSC. After exclusion of unrelated records included by the keyword search mode, the numbers of clinical trials on MSC include 96 studies of BM-MSC, 30 studies of UC-MSC, and 7 studies of UCB-MSC, as summarized in Table 4. Many trials may benefit from the anti-fibrotic effect [96] and

immune-modulation capacities [7] of MSC, but they also anticipate to validate the regeneration effect of MSC as well. The treated organs represent a wide range including eye, heart, liver, kidney, bone, cartilage, blood or blood vessel, etc. From the table it can be seen that the focuses of clinical interest of BM-MSC are for bone (18%), liver (15%), cartilage (14%) and heart (14%) repair, while currently UC-MSC are mainly tested for liver (23%) and neural (20%) disorders, and UCB-MSC are mainly tested for cartilage (29%) and lung (29%) diseases. Encouragingly, some of the clinical focus is in line with the characteristics of that specific type of MSC. For example, the relatively higher pancreagenesis and neurogenesis potential of UC-MSC may support their application in liver and neural disorders. Similarly, the application of BM-MSC in heart diseases is backed by their relatively higher cardiomyogenesis potential. However some other trials may not be so superior, such as using UCB-MSC for cartilage repair. In the future, more *in vitro* experimental work revealing the differences in the intrinsic nature of different types of MSC may serve as a guide for the design of clinical trials.

It is worrying that due to the lack of consensus markers of MSC, in conjunction with the lack of standardization of isolation techniques, experimental results can vary not only from one group to another, but also vary from one sample to another, even when they were prepared with the same protocol. This has been demonstrated in Markov's study [72], where significant differences in gene expressions were present among MSC from UCB-1 and UCB-2. Furthermore, culturing conditions and expansion period in culture may have a great influence on the gene profile of MSC, as reported by Jeong's group [96], when analyzing protein expression of two UCB-MSC populations from the same culture, one cell population at passage 3-5, the other population at passage 8, it turned out the hydrophobic proteomes of the two populations differed from each other. MSC proteome is intrinsically

sensitive to its environment and highly related to its functional state, so extreme caution should be taken for experimental design, for example, by keeping the cells in a same culture condition and using the same passage of cells for analysis purpose, to make the comparison truly comparable.

Conflict of interest

The authors declare no potential conflict of financial interest.

Acknowledgements

This study was partly supported by the Natural Science Foundation of China (81171746 and 81130034), Key Research Project of Shenzhen (JC201005250046A) and Research Grants Council of Hong Kong (HKU762707M).

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Figure Legends

Figure 1. Reanalysis of cytokine expression at mRNA and protein level in BM-MSC, UC-MSC and UCB-MSC. Top: mRNA level by microarray. In the original paper the signal intensity was presented in green/red color, and the standard bar ranged from -3.0 (green) to 3.0 (red). We normalized the signal of individual gene expression to the standard bar by Image-Pro Plus 5.1 to quantify the level of individual gene expression. The result represents the Mean±SEM of 3 batches of BM-MSC, 8 batches of UC-MSC, and 2 batches of UCB-MSC, respectively. Bottom: protein level by ELISA-based assay. The concentration of cytokines, expressed in the original paper by –, +, ++, +++, was quantified as 0, 50, 500, 5000 pg/ml. The result represents the mean of 3 batches of BM-MSC, UC-MSC and UCB-MSC, respectively. Notice the consistant changes in the two graphs of the expression level of IL-6, IL-8, IL-12 and IL-15 in UC-MSC and UCB-MSC, compared with BM-MSC.

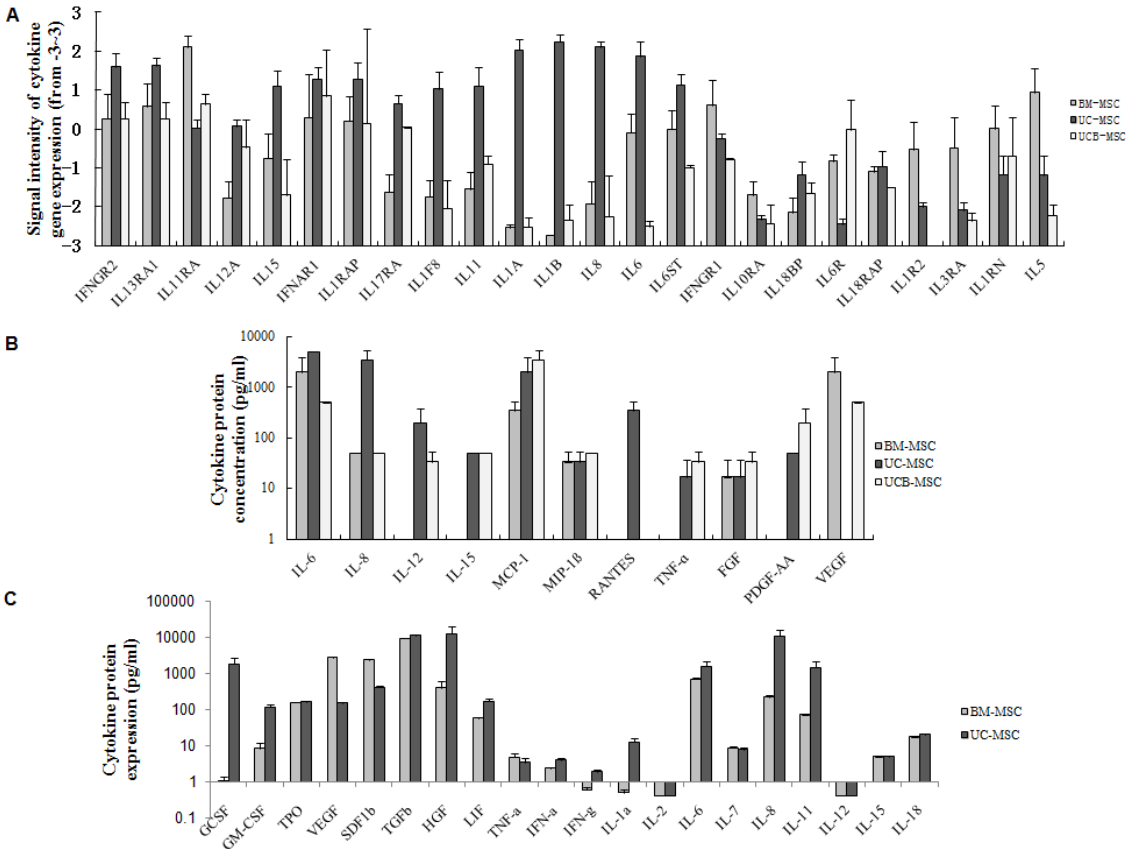
A
Signal intensity of cytokine gene expression (from -3-3)
3
2
1
0
-1
-2
-3
BM-MSC
UC-MSC
UCB-MSC
IFNGR2
IL13RA1
IL11RA
IL12A
IL15
IFNAR1
IL1RAP
IL17RA
IL1F8
IL11
IL1A
IL1B
IL8
IL6
IL6ST
IFNGR1
IL10RA
IL18BP
IL6R
IL18RAP
IL1R2
IL3RA
IL1RN
IL5
B
Cytokine protein concentration (pg/ml)
10000
1000
100
10
1
BM-MSC
UC-MSC
UCB-MSC
IL-6
IL-8
IL-12
IL-15
MCP-1
MIP-1ß
RANTES
TNF-a
FGF
PDGF-AA
VEGF
C
Cytokine protein expression (pg/ml)
100000
10000
1000
100
10
1
0.1
BM-MSC
UC-MSC
GCSF
GM-CSF
TPO
VEGF
SDF1b
TGFb
HGF
LIF
TNF-a
IFN-a
IFN-g
IL-1a
IL-2
IL-6
IL-7
IL-8
IL-11
IL-12
IL-15
IL-18

Table 1. Methodologies developed for UC-MSC isolation.

References	storage time before processing	Cord processing	Enzymatic digestion solution	Culture medium	Seeding density	Outcome
Part A	MSC isolation from Wharton's Jelly of UC					
[53, 54]	6~12 hr	The inner surface of the vessel was filled with enzymatic solution to collect endothelial and subendothelial cells	0.1% collagenase in Medium 199 at 37℃ for 15 minutes	DMEM-lowglucose (with 20 mM HEPES, 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine, 1 mM sodium pyruvate, and 10% FBS.)	10^3 cells/cm^2	Initial cultures contained mostly endothelial cells, which were 1 week later outgrown by fibroblast-like cells. A homogeneous layer of MSC-like cells formed by the third week. Success rate was low (3 out of 50 samples).
[52]	N/A	The whole UC was minced into 1~2 mm fragments and put into digestion solution	digest in 0.075% collagenase type II for 30 min and then 0.125% trypsin for 30 min with gentle agitation at 37℃	DMEM-low glucose (with and 5% FBS, 10 ng/mL VEGF, 10 ng/mL EGF，100 U penicillin/streptomycin, and 2mM L-glutamine.)	1×10^6 cells/cm^2	The mean yield of nucleated cells was 1×10^6/cm and the yield of adherent cells was 8.6×10^5/cm. By 2 weeks, a monolayer of homogenous spindle-like cells was formed.
[33]	1~24 hr	Connective tissue was scraped off the Wharton's Jelly after removal of blood vessels	digest in 2mg/ml collagenase for 16 hrs and 2.5% trypsin for 30min at 37℃ with agitation	DMEM (with 10% FBS and 4.5 g/L glucose.)		
[36, 52][70]	2~6 hr	First the cord was cut into 1.5cm pieces, the inner WJ surface was exposed to enzymatic solution for 45 min. Then the WJ was separated by the blunt surface of forceps and passed through a 18G needle.	collagenase type I, collagenase type IV and 100 IU of hyaluronidase in DMEM medium at 37℃ for 45 min	DMEM-high glucose (with 20% FBS, 16 ng/ml bovine FGF; 1 mM/L-glutamine, 1:200 dilution of ITS and antimycotic-antibiotic solution.)	$4.7x10^6$ live cells/cm^2	Success rate was 100% (13 out of 13 samples). Primary culture showed epitheliod-like morphology. Third passage cultures showed fibroblastic-like morphology.
[59]	<6 hr	The cord was cut into 1.5cm pieces, sectioned longitudinally to expose the WJ, and placed in culture medium. After 15 days, cells attached to culture surface.	No digestion.	DMEM low-glucose (with 10% FCS, 1xNEAA, 1x antibiotics–antimycotics, 2mM L-glutamine.)	one cord piece per well in 6-well plates	In first passage, the cells assumed a polymorphic, fibroblast-like morphology.
Part B	MSC isolation from perivascular region of UC					

[32]	N/A	The vessels of 4~5cm length were isolated with surrounding matrix, and the two ends were tied with surgical suture to form a loop. After digestion, the suspended cells were CD45 depleted.	1 mg/ml collagenase in PBS at 37℃ for 18-24 hours	75% α-MEM, 15% FBS, and 10% antibiotics	N/A	Average yield was $2\sim5\times10^6$ cells per cord, representing a harvesting yield of $2.5\sim25\times10^4$ cells/cm of cord and a success rate of 100% (n=72). The harvested cells exhibited a morphologically homogeneous "fibroblast-like" appearance.
[28]		The vessels of 1cm length were subjected to enzymatic solution.	an enzyme cocktail for 3 h at 37℃.			The cells could be isolated in sufficient quantities and demonstrated a fibroblast-like phenotype.
[66]	<24 hr	Cord pieces of 0.5~1cm were removed of vessels and minced, then cryopreserved in 10% DMSO and heat-inactivated autologous cord plasma	No digestion.	RPMI-1640 (with 20% FBS, penicillin 100 mg/ml, streptomycin 10mg/ml, amphote ricin B 250mg/ml, and ciprofloxacin 10 mg/ml)	N/A	The viability of thawed UC-MSC was consistently > 90%. There was also no difference as to whether cells were initially cryopreserved in either VueLife bags or cyrovials. The mean doubling time was 2.26 days.
[67]	1~5 days	Cord pieces of 3~5cm pre-rinsed in 75% ethanol for 30s, removed of vessels, minced into 0.5 – 1mm 2 pieces,	No digestion.	DMEM-low glucose (with 10% FBS, 2 mM L-glutamine, 100 U/ml penicillin, 100μg/ml streptomycin, 25μg/ml Fungizone, 5ng/ml basic FGF and 5ng/ml EGF.)	1 cord per 100×20 mm petri dish	Cell yield was $1.1\sim5.0\times10^6$ cells/cord at 21~30d after processing. Cells can be derived 4 and 5 days following the sample collection, but the cell yield decreased. 1d: $2.50 \pm 0.20 \times 10^6$; 2d: $1.30 \pm 0.18 \times 10^6$;3d:$1.20 \pm 0.14 \times 10^6$; 4d: $0.84 \pm 0.07 \times 10^6$; 5d: $0.74 \pm 0.10 \times 10^6$

FBS: fetal bovine serum. FCS: fetal calf serum. VEGF: vascular endothelial growth factor. EGF: epidermal growth factor. FGF: fibroblast growth factor. ITS: insulin-transferrin-selenium. NEAA: non-essential aminoacids. PBS: with phosphate buffered saline. N/A: not available.

Table 2. Comparison of multipotency of BM-MSC, UC-MSC, UCB-MSC and AT-MSC.

	BM-MSC	UC-MSC	UCB-MSC	AT-MSC
Adipogenesis	++	+ [61]	-~+ [18, 41, 77]	
Chondrogenesis	++	+++ [84]	+ [72]	+ [72]
Osteogenesis	++	+++ [75, 79] or + [61] [80]	+++ [77]	- [81] or ++ [82]
Endothelial differentiation	++	+++ [74]		
Pancreagenesis	++	+++ [73]		
Neurogenesis	++	+++ [52]		
Cardiamyogenesis	++	- [61]	- [61]	

Enhanced, reduced: represents enhanced or reduced differentiation potential compared with BM-MSC.
-: differentiation not detected, ++: differentiation potential of BM-MSC, +++: higher differentiation potential than BM-MSC, +: differentiation detected but lower than BM-MSC.

Table 3. Surface protein expression of BM-MSC, UC-MSC, UCB-MSC and AT-MSC.

Proteins	References	BM-MSC	UC-MSC	UCB-MSC	AT-MSC
CD106	[61]	+	-	+	
	[52]	+++	+	-	
	[42]	++~+++			-
	[90]	+~++			-
	[18]	+++		+++	++
CD49d	[91]	-			+
	[90]	-			-/+
	[40]			+	
	[42]				+++
	[97]		-/+		
CD54	[91]	+			+++
CD34	[61]	-	-	-	
	[91]	-			-/+~+
	[42]	-	-	-	
HLA-ABC	[52]	++++	+++		
	[42]	++++		++	++
	[18]	++++		++++	++++
	[66]	++	+		
HLA-G	[59]		+		
CD146	[61]	++	++	++++	
	[98]				+

The comparison of protein expression in each reference was performed with the same antibody and staining protocol. -~++++ represent the percentage of positive subsets in the given MSC population. -: 0~1%, -/+: 1~5%, +: 5~25%, ++: 25~50%, +++: 50~75%, ++++:>75%.

Table 4. Summary of clinical trials using BM-MSC, UC-MSC and UCB-MSC.

Disease classification	BM-MSC		UC-MSC		UCB-MSC	
	No.of trials	Percentage	No.of trials	Percentage	No.of trials	Percentage
Eye	2	2%		0%		0%
liver	14	15%	7	23%		0%
kidney	1	1%	1	3%		0%
lung	1	1%	1	3%	2	29%
bone	17	18%	1	3%	0	0%
cartilage	13	14%	2	7%	2	29%
muscle		0%	1	3%		0%
skin	2	2%	1	3%		0%
blood vessel	5	5%		0%		0%
Heart disease	13	14%	1	3%		0%
Gastrointestinal disease	2	2%	1	3%		0%
Diabetes	6	6%	4	13%		0%
HSC/blood cell support		0%	1	3%	1	14%
Limb ischemia	3	3%		0%		0%
neural disorder	7	7%	6	20%	1	14%
blood disorders	3	3%	2	7%		0%
graft rejection	7	7%	1	3%	1	14%
Total	96		30		7	