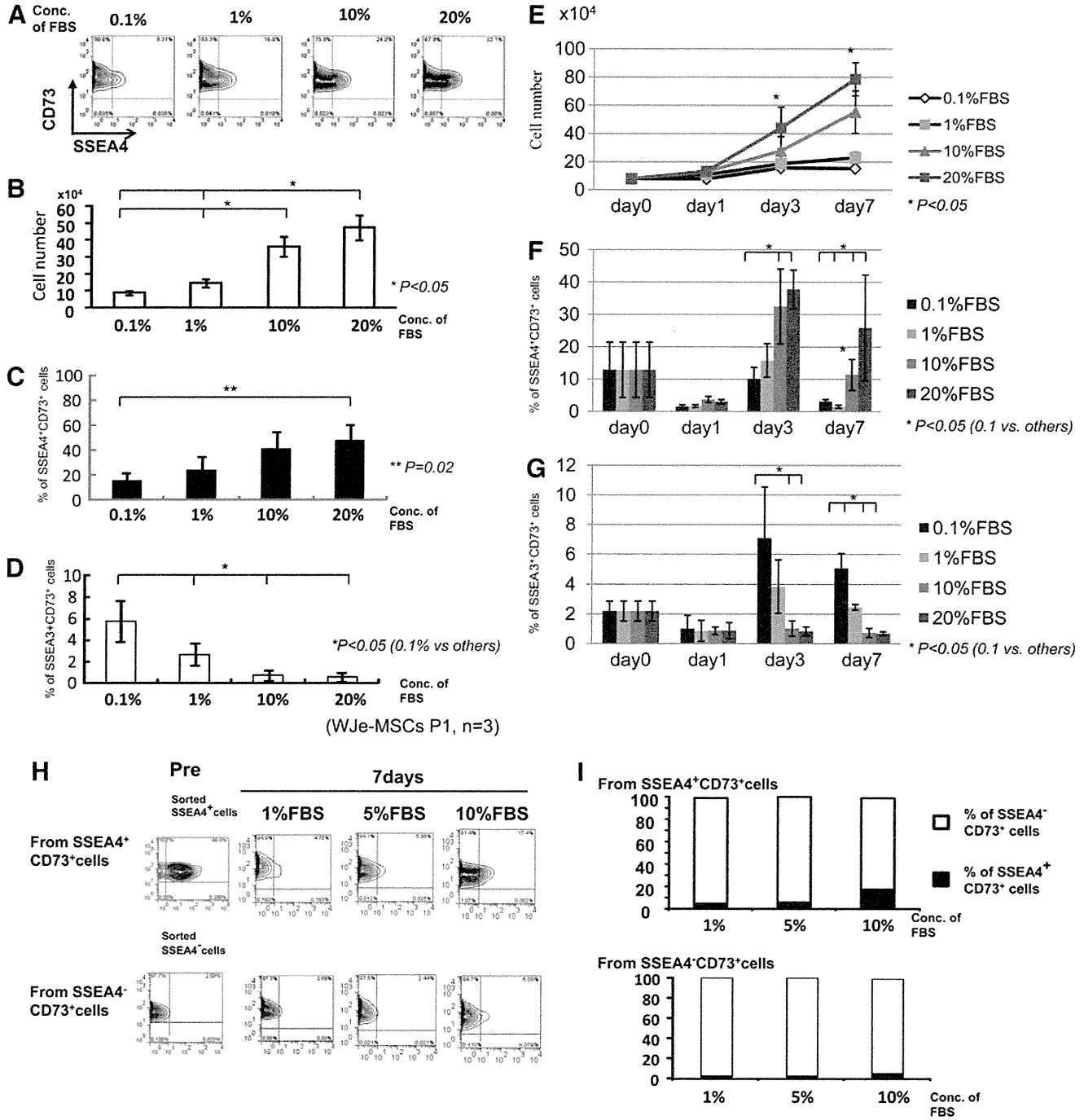

FIG. 6. Influence of fetal bovine serum (FBS) on SSEA4 and SSEA3 expression. **(A)** WJ-MSCs collected by the explant method (WJe-MSCs) were cultured for 1 week in media containing 0.1%, 1%, 10%, and 20% FBS, and SSEA4 expression patterns were analyzed by flow cytometry. The data are representative of three individual experiments. **(B)** WJe-MSC numbers in media with different concentrations of FBS were determined. **(C, D)** Percentages of $SSEA4^+$ and $SSEA3^+$ cells, respectively, among the WJe-MSCs in media with different concentrations of FBS were determined. **(E)** Growth curves of WJe-MSCs with different concentrations of FBS. **(F, G)** Percentage of $SSEA4^+$ and $SSEA3^+$ cells during the proliferation of WJe-MSCs, respectively. **(H, I)** Flow cytometry patterns and percentages of SSEA4 expression in the sorted $SSEA4^+$ and $SSEA4^-$ WJe-MSCs in media with 1%, 5%, and 10% FBS were determined. $^*P<0.05$, $^{**}P=0.02$.

cells with low incidence of MSCs and also low incidence of SSEA4+cells. As expected, the proportion of $CD45^+$ hematopoietic cell expression declined during the passages; instead, $CD45^-$ cells increased (Fig. 7A). The latter cells expressed $CD45^-CD73^+$ MSCs (data not shown). However, in α-MEM with 10% FBS, we could not observe the predominant growth of $SSEA4^+$ cells regardless of CD45 expression (Fig. 7A). Further, in consistent with the results in WJ-MSCs, the SSEA4 expression in BM-MSCs was correlated with the FBS concentration, whereas SSEA3

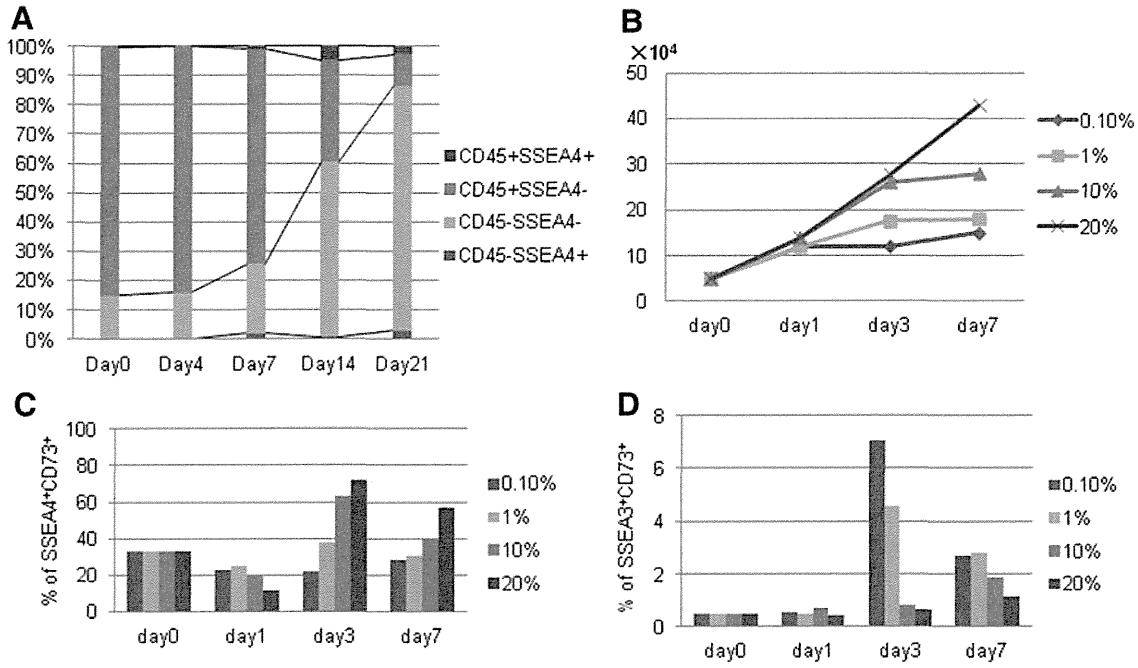

FIG. 7. Influence of FBS on SSEA4 and SSEA3 expression in bone-marrow-derived mesenchymal stem cells. **(A)** Shift of the proportion of $CD45^+$ hematopoietic cells and $CD45^-$ cells with or without SSEA4 expression during the passages. **(B)** Growth curves of BM-MSCs with different concentrations of FBS. **(C, D)** Percentage of $SSEA4^+$ and $SSEA3^+$ cells during the proliferation of BM-MSCs, respectively. The data are representative of two individual experiments.

expression was inversely correlated (Fig. 7B–D). The relationship between the growth curve and SSEA4/3 expression was also consistent with the results in WJ-MSCs (Figs. 6E–G and 7B–D).

Discussion

To gain insight into the role of SSEA3 and SSEA4 in UC WJ-MSCs, we examined the SSEA3 and SSEA4 expression on WJ-MSCs obtained by different methods and compared the differentiation abilities of $SSEA4^+$ and $SSEA4^-$ cells.

First, we compared SSEA3 and SSEA4 expression in WJe-MSCs and WJc-MSCs. The explant method has several advantages. It does not require nonhuman collagenase derived from *Clostridium histolyticum* and saves the time for lysing the tissue with enzyme in the procedure. The disadvantages of the explant method are that collecting adequate cell numbers is dependent on the amount of WJ tissue fragments that attach firmly to the bottom of the dish, which in turn, is dependent on the individual ability. Although the collagenase process from cutting tissue to plating can be unified, we found that some samples are more sensitive to collagenase, resulting in reduced viability. We did not find any significant differences between WJe-MSCs and WJc-MSCs in cell numbers, MSC surface markers as defined by the ISCT, or ES-cell-related gene expression at P0. In addition, the incidence of $SSEA4^+$ and $SSEA3^+$ at P0 was similar between the two types. However, SSEA3 disappeared rapidly in the early culture passages, as described previously.[14,20] It is known that SSEA3 disappears more rapidly from the cell surface compared with SSEA4, if GSL synthesis is blocked by inhibitors, and a similar phenomenon has also been detected during ES cell differentiation.[14,21] Interestingly, the incidence of WJe-MSC $SSEA4^+$ cells was reduced after the first passage, and, by P7, the original levels were recovered. In contrast, the incidence of WJc-MSC $SSEA4^+$ cells was relatively stable until P9. The reason for the depression of SSEA4 expression in WJe-MSCs at P1 is unknown. However, $SSEA4^+$ MSCs in both WJe-MSCs and WJc-MSCs did not proliferate predominantly in our culture medium. As previously reported, osteogenic differentiation was difficult with WJ-MSCs,[8] requiring a longer induction period, additional cytokines such as BMP2, and specific FBS concentrations. However, both sorted $SSEA4^+$ and $SSEA4^-$ WJ-MSCs eventually differentiated into osteocytes and adipocytes in a similar manner, and there were no differences in ES-marker gene expression between the $SSEA4^+$ and $SSEA4^-$ MSCs. Interestingly, $SSEA4^+$ cells appeared even from the $SSEA4^-$ MSCs, and the incidence of $SSEA4^+$ cells derived from the $SSEA4^-$ MSCs demonstrated a similar transition pattern as those derived from the $SSEA4^+$ MSCs. This result suggested that the culture medium may have been the source of SSEA4 antigens.

The role of SSEA3 and SSEA4 in MSCs remains controversial. Rostovskaya *et al.* also suggested SSEA4-marked adipogenic progenitor lacking osteogenic capacity.[22] In ES cell study, Ramirez *et al.* demonstrated that both SSEA3 and SSEA4 are markers of immature ES cells, but particularly SSEA3 together with OCT4 and TRA-1–60 were good tracers for validating pluripotent stem cells, whereas SSEA4 was expressed for long during the differentiation of ES cells.[20] Gang *et al.* reported that $SSEA4^+$ cells proliferated predominantly when the culture was initiated from primary BM cells, which were mostly hematopoietic cells.[11] But in our culture condition, the incidence of $SSEA4^+$ cells, which were characterized as MSCs, was not increased dramatically

as reported by Gang's group. Their results might be induced by the special cocktail of the medium, consisted of MCDB-201, 10% FBS, ITS, linoleic acid–bovine serum albumin, dexamethasone, ascorbic acid, hPDGF-BB, and hEGF, followed by the medium with relatively high FBS concentration. In other articles, Schrobback *et al.* assessed the SSEA4 in human articular chondrocytes, osteoblasts, and BM-derived MSCs and characterized their differentiation potential. But their results showed that SSEA4 levels in these cells were not unrelated to the cells' chondrogenic and osteogenic and proliferation potentials *in vitro*.[23] Suila *et al.* reported that SSEA4, and not SSEA3, was expressed on the surface of cord-blood-derived MSCs, whereas SSEA3 was expressed at very low levels in cord blood hematopoietic stem cells.[24] They also suggested that FBS contains detectable amounts of globoseries GSLs and showed that the SSEA3 was influenced and upregulated by culturing with FBS overnight, even though they did not demonstrate an influence on SSEA4 expression. In our study, we demonstrated that SSEA4 expression significantly correlated with FBS concentration, whereas SSEA3 appeared to be negatively correlated with FBS concentration. The possibility that FBS stimulated the proliferation of WJe-MSCs, resulting in the increase of SSEA4 expression, cannot be denied completely. But the fact that the SSEA4 expression was declined during the proliferation of WJe-MSCs and BM-MSCs in each medium did not support this hypothesis. Regardless of cell growth or cell concentration, consistently, the SSEA4 expression was clearly associated with FBS concentration not only in WJ-MSCs but also in BM-MSCs. Reversely to the SSEA4 expression, SSEA3 expression appeared to be negatively correlated with FBS concentration. The reason why the data of SSEA3 elevation upon the higher FBS concentration are not coincident with Suila's data remained unresolved. We add a partial speculation that SSEA4 is derived from SSEA3; thus, the increase of SSEA4 means the waste of SSEA3, or MSCs with high concentration of FBS are differentiated accompanied with decrease of SSEA3. But we need the scrupulous attention to analyze SSEA3 by FCM in various FBS concentrations. We also found that SSEA4 could be induced from pure $SSEA4^-$ WJ-MSCs. This suggests the presence of substrate for SSEA4 in $SSEA4^-$ cells and also the influence of FBS on the SSEA4 expression in $SSEA4^-$ cells. Brimble *et al.* reported that the depletion of these two molecules by the addition of GSL synthesis inhibitors apparently did not affect the ES cell pluripotency.[14] In conclusion, these results indicate that SSEA4 may display altered expression profiles in response to culture medium including FBS and may not be an essential marker of WJ-MSC pluripotency.

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Disclosure Statement

The authors have no conflicts of interest.

References

1. Ishige, I., Nagamura-Inoue, T., Honda, M.J., Harnprasopwat, R., Kido, M., Sugimoto, M., *et al.* Comparison of mesenchymal stem cells derived from arterial, venous, and Wharton's jelly explants of human umbilical cord. Int J Hematol **90,** 261, 2009.
2. Kode, J.A., Mukherjee, S., Joglekar, M.V., and Hardikar, A.A. Mesenchymal stem cells: immunobiology and role in immunomodulation and tissue regeneration. Cytotherapy **11,** 377, 2009.
3. Zeddou, M., Briquet, A., Relic, B., Josse, C., Malaise, M.G., Gothot, A., *et al.* The umbilical cord matrix is a better source of mesenchymal stem cells (MSC) than the umbilical cord blood. Cell Biol Int **34,** 693, 2010.
4. Horwitz, E.M., Le Blanc, K., Dominici, M., Mueller, I., Slaper-Cortenbach, I., Marini, F.C., *et al.* Clarification of the nomenclature for MSC: The International Society for Cellular Therapy position statement. Cytotherapy **7,** 393, 2005.
5. Dominici, M., Le Blanc, K., Mueller, I., Slaper-Cortenbach, I., Marini, F., Krause, D., *et al.* Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. Cytotherapy **8,** 315, 2006.
6. Anzalone, R., Iacono, M.L., Corrao, S., Magno, F., Loria, T., Cappello, F., *et al.* New emerging potentials for human Wharton's jelly mesenchymal stem cells: immunological features and hepatocyte-like differentiative capacity. Stem Cells Dev **19,** 423, 2010.
7. Carvalho, M.M., Teixeira, F.G., Reis, R.L., Sousa, N., and Salgado, A.J. Mesenchymal stem cells in the umbilical cord: phenotypic characterization, secretome and applications in central nervous system regenerative medicine. Curr Stem Cell Res Ther **6,** 221, 2011.
8. Hsieh, J.Y., Fu, Y.S., Chang, S.J., Tsuang, Y.H., and Wang, H.W. Functional module analysis reveals differential osteogenic and stemness potentials in human mesenchymal stem cells from bone marrow and Wharton's jelly of umbilical cord. Stem Cells Dev **19,** 1895, 2010.
9. Fong, C.Y., Chak, L.L., Biswas, A., Tan, J.H., Gauthaman, K., Chan, W.K., *et al.* Human Wharton's jelly stem cells have unique transcriptome profiles compared to human embryonic stem cells and other mesenchymal stem cells. Stem Cell Rev **7,** 1, 2011.
10. Kannagi, R., Cochran, N.A., Ishigami, F., Hakomori, S., Andrews, P.W., Knowles, B.B., *et al.* Stage-specific embryonic antigens (SSEA-3 and -4) are epitopes of a unique globo-series ganglioside isolated from human teratocarcinoma cells. EMBO J **2,** 2355, 1983.
11. Gang, E.J., Bosnakovski, D., Figueiredo, C.A., Visser, J.W., and Perlingeiro, R.C. SSEA-4 identifies mesenchymal stem cells from bone marrow. Blood **109,** 1743, 2007.
12. Kuroda, Y., Kitada, M., Wakao, S., Nishikawa, K., Tanimura, Y., Makinoshima, H., *et al.* Unique multipotent cells in adult human mesenchymal cell populations. Proc Natl Acad Sci USA **107,** 8639, 2010.
13. Wakao, S., Kitada, M., Kuroda, Y., and Dezawa, M. Isolation of adult human pluripotent stem cells from mesenchymal cell populations and their application to liver damages. Methods Mol Biol **826,** 89, 2012.
14. Brimble, S.N. Sherrer, E.S., Uhl, E.W., Wang, E., Kelly, S., Merrill, A.H. Jr., Robins, A.J., and Schulz, T.C. The cell surface glycosphingolipids SSEA-3 and SSEA-4 are not essential for human ESC pluripotency. Stem Cells **25,** 54, 2007.

15. Tong, C.K., Vellasamy, S., Tan, B.C., Abdullah, M., Vidyadaran, S., Seow, H.F., *et al.* Generation of mesenchymal stem cell from human umbilical cord tissue using a combination enzymatic and mechanical disassociation method. Cell Biol Int **35,** 221, 2011.
16. Lee, K.S., Nah, J.J., Lee, B.C., Lee, H.T., Lee, H.S., So, B.J., *et al.* Maintenance and characterization of multipotent mesenchymal stem cells isolated from canine umbilical cord matrix by collagenase digestion. Res Vet Sci **94,** 144, 2013.
17. Takahashi, K., Tanabe, K., Ohnuki, M., Narita, M., Ichisaka, T., Tomoda, K., *et al.* Induction of pluripotent stem cells from adult human fibroblasts by defined factors. Cell **131,** 861, 2007.
18. Wulsten, D., Glatt, V., Ellinghaus, A., Schmidt-Bleek, K., Petersen, A., Schell, H., *et al.* Time kinetics of bone defect healing in response to BMP-2 and GDF-5 characterised by *in vivo* biomechanics. Eur Cells Mater **21,** 177, 2011.
19. Agata, H., Asahina, I., Watanabe, N., Ishii, Y., Kubo, N., Ohshima, S., *et al.* Characteristic change and loss of *in vivo* osteogenic abilities of human bone marrow stromal cells during passage. Tissue Eng Part A **16,** 663, 2010.
20. Ramirez, J.M., Gerbal-Chaloin, S., Milhavet, O., Qiang, B., Becker, F., Assou, S., *et al.* Brief report: benchmarking human pluripotent stem cell markers during differentiation into the three germ layers unveils a striking heterogeneity: all markers are not equal. Stem Cells **29,** 1469, 2011.
21. Draper, J.S., Pigott, C., Thomson, J.A., and Andrews, P.W. Surface antigens of human embryonic stem cells: changes upon differentiation in culture. J Anat **200,** 249, 2002.
22. Rostovskaya, M., and Anastassiadis, K. Differential expression of surface markers in mouse bone marrow mesenchymal stromal cell subpopulations with distinct lineage commitment. PloS One **7,** e51221, 2012.
23. Schrobback, K., Wrobel, J., Hutmacher, D.W., Woodfield, T.B., and Klein, T.J. Stage-specific embryonic antigen-4 is not a marker for chondrogenic and osteogenic potential in cultured chondrocytes and mesenchymal progenitor cells. Tissue Eng Part A **19,** 1316, 2013.
24. Suila, H., Pitkanen, V., Hirvonen, T., Heiskanen, A., Anderson, H., Laitinen, A., *et al.* Are globoseries glycosphingolipids SSEA-3 and -4 markers for stem cells derived from human umbilical cord blood? J Mol Cell Biol **3,** 99, 2011.

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TOPIC HIGHLIGHT

WJSC 6th Anniversary Special Issues (2): Mesenchymal stem cells

Umbilical cord-derived mesenchymal stem cells: Their advantages and potential clinical utility

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Abstract

Human umbilical cord (UC) is a promising source of mesenchymal stem cells (MSCs). Apart from their prominent advantages, such as a painless collection procedure and faster self-renewal, UC-MSCs have shown the ability to differentiate into three germ layers, to accumulate in damaged tissue or inflamed regions, to promote tissue repair, and to modulate immune response. There are diverse protocols and culture methods for the isolation of MSCs from the various compartments of UC, such as Wharton's jelly, vein, arteries, UC lining and subamnion and perivascular regions. In this review, we give a brief introduction to various compartments of UC as a source of MSCs and emphasize the potential clinical utility of UC-MSCs for regenerative medicine and immunotherapy.

Key words: Umbilical cord; Mesenchymal stem cells; Wharton's Jelly; Multipotency; Immunotherapy

Core tip: Human umbilical cord (UC) is a promising source of mesenchymal stem cells (MSCs). UC-MSCs have shown the ability of faster self-renewal and to differentiate into three germ layers, to accumulate in damaged tissue or inflamed regions, to promote tissue repair, and to modulate immune response. There are diverse protocols and culture methods for the isolation of MSCs from the various compartments of UC, such as Wharton's jelly, vein, arteries, UC lining membrane and subamnion and perivascular regions. In this review, we introduce various compartments of UC and discuss the potential clinical utility of UC-MSCs for regenerative medicine and immunotherapy.

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INTRODUCTION

Mesenchymal stem cells (MSCs) originate in the human embryo and are considered multipotent stem cells. MSCs are a heterogeneous subset of stromal stem cells, which can be isolated from the bone marrow[1], mobilized peripheral blood[2], cord blood[3], umbilical cord (UC)[4,5], placenta[6], adipose tissue[7], dental pulp[8], and even the fetal liver[9] and lungs[10]. UC contains two umbilical arteries (UCAs) and one umbilical vein (UCV), both embedded within a specific mucous connective tissue, known as Wharton's jelly (WJ), which is covered by amniotic epithelium (Figure 1). UC is considered medical waste and the collection of UC-MSCs is noninvasive; furthermore, the access to UC-MSCs has not been encumbered with ethical problems. UC-MSCs, similarly to MSCs derived

from other sources, have distinct capacity for self-renewal while maintaining their multipotency, *i.e.*, the ability to differentiate into adipocytes, osteocytes, chondrocytes, neurons and hepatocytes, although some differentiation abilities are known to be partial[11-13]. Moreover, UC-MSCs have also attracted great interest because of their immunomodulatory properties. Nowadays, UC-MSCs are proposed as a possible versatile tool for regenerative medicine and immunotherapy.

HISTORY OF UC-MSCs

During pregnancy, the fetus and mother are connected by UC. UC prevents umbilical vessels from compression, torsion and bending, while providing good blood circulation. McElreavey *et al*[4] for the first time reported isolation of fibroblast-like cells from WJ of human UC in 1991. The UC-derived cells have the similar surface phenotype, plastic adherence and multipotency as those of MSCs derived from other sources. It was 3 years earlier that the first cord blood (CB) transplantation was performed in France in 1988[14]. After that, together with the development of CB banking, CB transplantation has become the alternative source of hematopoietic stem cells. Although CB-derived MSCs cannot be consistently isolated[15-18], MSCs were considered to circulate in the blood of preterm fetuses and able to be successfully isolated and expanded[3]. Where these cells home at the end of gestation is not clear[13]. Thus, UC has inevitably become a focus of attention as a source of MSCs because it contains CB[18]. One key study appeared concerning CB-derived MSCs appeared around 2003[19]. Mitchell *et al*[20] successfully isolated matrix cells from UC WJ using explant culture and Romanov *et al*[19] isolated MSCs-like cells from the subendothelial layer of UCV.

ADVANTAGES OF UC-MSCs

Stem cell populations can be isolated from embryonic, fetal and adult tissues. Embryonic stem cells (ESCs) are a leading candidate for tissue engineering because of their high self-renewal capacity and pluripotency (ability to differentiate into all germ layers) *in vitro* and *in vivo*. Nonetheless, in addition to ethical restrictions, the clinical applications of ESCs are severely limited by technical difficulties with the depletion of immature cells that may result in the formation of a teratoma.

In contrast, adult stem cells, such as those in the skin, bone marrow (BM) and adipose tissue, may have wider clinical applications. BM-MSCs have been used for autologous and allogeneic purposes. Recently, successful clinical application of autologous BM-MSCs was reported for conditions such as cardiac infarction[21], graft-versus-host disease (GVHD)[22,23], Crohn's disease[24] and bone tissue engineering[25]. On the other hand, the autologous use was sometimes limited by cell numbers and age-related changes such as decreased growth and differentiation capacity[26,27].

Compared with BM-MSCs and ES cells, UC-MSCs show a gene expression profile more similar to that of ESCs and faster self-renewal rather than BM-MSCs[11,12].

It is easy to obtain a substantial number of UC-MSCs after several passages and extensive *ex vivo* expansion[28]. The most appreciable advantage is that the collection procedure is noninvasive and ethically acceptable.

Similar to BM-MSCs, UC-MSCs can be considered for autologous and allogeneic use. Autologous UC-MSCs might be used as gene therapy for genetic diseases and as regenerative or anti-inflammatory therapy for neonatal injury, such as cerebral palsy or hypoxic brain damage. On the other hand, allogeneic UC-MSCs can be expanded and cryopreserved in a cell bank for patients in need. The only disadvantage is that physicians need to confirm the baby's health as a donor because it cannot be ascertained in advance whether the donor will grow normally without health problems; thus, genomic or chromosomal tests need to be performed. In contrast, in the case of a BM donor, the physician can directly see and examine the donor and then decide to collect BM. In the case of CB banking, many CB banks monitor the baby's health after birth. Thus, it is important to know the advantages and disadvantages of UC-MSCs for each clinical application.

DIFFERENT METHODS FOR ISOLATION OF MSCs FROM DIFFERENT COMPARTMENTS OF UC

There are two methods to obtain MSCs from various UC compartments or from the whole UC: the explant method and the enzymatic digestion method.

The explant method

UC, or its compartments, is manually minced into small fragments 1-2 mm^3. These fragments are aligned and seeded regularly on the tissue culture-treated dishes. After the tissue fragments are attached to the bottom of the dish, the culture medium is poured slowly and gently, so as not to detach the fragments, and the culture is started[29-31]. The culture medium is replaced every 3-7 d for 2-4 wk until fibroblast-like adherent cells reach 80%-90% confluence. The adherent cells and tissue fragments are rinsed once with PBS and detached using a trypsin solution, followed by washing with the medium. The cells and tissue fragments are filtered to remove the tissue fragments.

The disadvantage of this method is that the fragments often float in the medium, resulting in poor cell recovery. No MSCs can be obtained from the floating fragments. To collect a consistent number of MSCs each time, it is important to prevent the exfoliation of the tissue fragments from the bottom of plastic dishes.

The enzymatic digestion method

WJ is either directly exposed to enzymatic solutions to

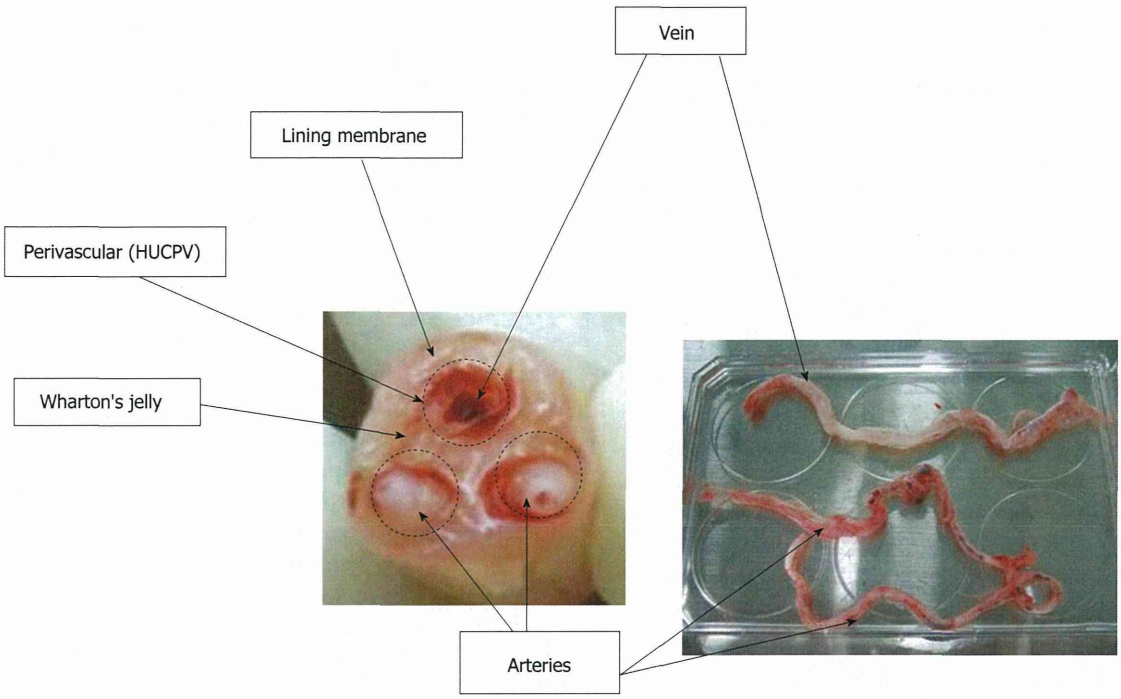

Figure 1 Various compartments of umbilical cord from which mesenchymal stem cells can be isolated. HUCPV: Human umbilical cord perivascular.

release the cells or it is cut into small pieces followed by enzymatic digestion. The enzymes used for digestion vary from simple collagenase[31,32] to a combination of either collagenase and hyaluronidase with or without trypsin[33,34] or collagenase, dispase II and hyaluronidase[33]. The digestion time and concentrations varied by researchers.

There are four compartments of UC as a source of MSCs: (1) Whole UC-MSCs: the whole UC is cut into smaller pieces followed by either an explant procedure or enzyme digestion[30,35,36]; (2) UCWJ-, UCA- and UCV-MSCs: UCWJ-MSCs are obtained after removing umbilical vessels. Umbilical vessels [two arteries (UCAs) and one vein (UCV)] can also be minced into 1-2 mm^3 fragments. The fragments are aligned regularly on the plate and cultured until MSCs start growing; (3) UC lining and subamnion-derived MSCs: the subamnion region of UC lining membrane is removed with a razor blade and then cut into small pieces. These fragments are plated in plastic culture dishes until MSCs start growing (explant method). With this method, however, it might be difficult to remove the adjacent region underneath the amniotic epithelium completely[37,38]; and (4) Human UC perivascular stem cells (HUCPVC): the vessels are extracted from UC and tied at both ends into loops. The loops are then placed into an enzymatic solution for a defined period of time to allow the cells to separate from the perivascular region. The detached cells are cultured and collected as HUCPVCs[26,28,39].

It is still controversial whether the isolation of the cells from the whole or some compartment is superior to others with respect to their proliferation ability, differentiation ability and immunosuppressive capacity.

Proliferation assays

The frequency of colony-forming unit fibroblasts (CFU-F) is significantly higher in whole UC-derived MSCs than in BM-MSCs with limiting dilution[26,30,40]. The authors first compared UCWJ-MSCs, UCA-MSCs and UCV-MSCs. UCV-MSCs exhibited a significantly higher frequency of CFU-F than UCWJ-MSCs and UCA-MSCs, but the doubling time was not different among these cell types[5]. The Mennan group also reported that there are no significant differences among the various compartments of UC, although the cells derived from any UC compartment proliferate significantly faster than BM-MSCs, with mean doubling times of 2-3 d at P0 through P3[41]. Depending on the purpose, researchers need to select either a compartment or the whole UC.

Biomarkers of UC-MSCs

The immunoprofile of UC-MSCs is analyzed using flow cytometry, according to the standard definitions for MSCs described by the position paper of the International Society for Cellular Therapy[42]. There are no single specific markers that can be used to identify multipotent MSCs. MSCs are positive for adhesion markers such as CD29 and CD44; mesenchymal markers such as CD90, CD73 and CD105; and human leukocyte antigen class I (HLA-ABC). MSCs are negative for endothelial cell marker CD31; hematopoietic cell markers such as CD34, CD45 and CD117; and human leukocyte differentiation antigen class II (HLA-DR)[43]. Among the different UC compartments, UCWJ-, UCV- and UCA-derived MSCs show a similar fibroblast-like spindle shape and the MSCs from these three types of tissues demonstrate no significant differences in the immunoprofile. These cells are positive for CD13, CD29 (integrin β1), CD73 (SH3), CD90 (Thy-1), CD105 (SH2; endogrin) and HLA-ABC at the cellular frequency greater than 90% and are negative for CD34,

CD45, CD133 and HLA-DR, with the cellular frequency less than 1%[5]. Mennan *et al*[41] also confirmed that MSC immunophenotypes showed no significant differences among different sources: BM, umbilical cord arteries, vein, WJ and UC lining membrane. Even although the authors could not find any major differences in their immunophenotypes, the cell populations derived from the different compartments may consist of different proportions of multipotent stem cells. Karahuseyinoglu *et al*[44] demonstrated that CD73 is expressed throughout the vessels and endothelium and is absent in the perivascular region, but the strongest expression is observed in the epithelial and subepithelial regions of WJ. CD90 is positive in most compartments but negative in the endothelial lumen lining. A high expression of vimentin, CKs (1, 4, 5, 6, 8, 10, 13, 18 and 19), desmin and SMA has been detected in the subamniotic layer and the perivascular region. Schugar *et al*[45] reported that CD146 (endothelial progenitor marker) is expressed in the vessel walls (100%) and the perivascular region of UC (62%) but is no longer expressed in UCWJ-MSCs[26,46]. These markers might aid in determining the multipotency of the isolated cell population. Phenotypic characterization of UC-MSCs might be influenced by the culture passage number, culture medium and culture method.

Furthermore, ESC markers such as Oct4, Nanog, Sox-2 and KLF4 are expressed only at low levels in UC-MSCs[47]. This suggests that MSCs are primitive stem cells, somewhere between ESCs and mature adult stem cells. Nonetheless, a precise isolation of pluripotent MSCs using specific markers remains a challenge.

The role of SSEA3 and SSEA4 in MSCs remains controversial. Gang *et al*[48] reported that $SSEA4^+$ cells proliferate predominantly when the culture is initiated from primary BM cells in the medium supplemented with special cocktails of cytokines. In contrast, the authors failed to reproduce the same phenomena in UCWJ-MSCs in the medium consisting of α-MEM and 10% FBS. Furthermore, SSEA4 expression in UCWJ-MSCs significantly correlates with the FBS concentration in the culture medium, whereas SSEA3 expression was inversely correlated. We concluded that SSEA4 in UCWJ-MSCs is not a marker of either proliferation capacity or multipotency[31]. Schrobback *et al*[49] assessed SSEA4 expression in human articular chondrocytes, osteoblasts and BM-derived MSCs and characterized their differentiation potential. Their results showed that SSEA4 levels in these cells are not related to the capacity for chondrogenic and osteogenic differentiation and the proliferation potential *in vitro*[49].

THE ABILITY OF UC-MSCs TO DIFFERENTIATE INTO ADIPOGENIC, CHONDROGENIC AND OSTEOGENIC LINEAGES

UC-MSCs originating from the extraembryonic mesoderm and their capacity for differentiation into adipogenic, chondrogenic and osteogenic lineages have been extensively studied[50]. Regarding the osteogenic differentiation ability, Hsieh *et al*[11] demonstrated that the gene profiles of UC-MSCs are close to ESCs; UC-MSCs show delayed and insufficient differentiation into osteocytes. On the other hand, BM-MSCs have already expressed an osteogenic gene profile and can easily differentiate into osteocytes. Among the three compartments, UCWJ, UCV and UCAs, UCWJ-MSCs demonstrate an obviously defective ability to differentiate into osteocytes, even although the expression of osteocyte-related genes is detected by reverse-transcriptase PCR, at levels similar to those in the other two tissues/compartments[5]. Mennan *et al*[41] compared the osteogenic differentiation among cord regions in six samples and found that the best differentiation is seen with UCWJ-MSCs and whole UC-derived MSCs, rather than with UCA-, UCV- and UC lining MSCs.

As for adipocytic differentiation, Mennan *et al*[41] reported that UC-MSCs produce small lipid vacuoles, whereas BM-MSCs produce more mature adipocytes (unilocular lipid vacuoles). UC-MSCs might maintain their multipotency for longer periods than BM-MSCs can[51], although there were no obvious differences among MSCs derived from UC compartments in our research[5].

With respect to chondrogenic differentiation, UC-MSCs show no apparent differences among the different cord regions (sources)[41]. Moreover, the comparison of the chondrogenic potential between BM-MSCs and UC-MSCs revealed that UC-MSCs produce thrice as much collagen as BM-MSCs; this finding indicates that the former may be a better option for fibrocartilage tissue engineering[52].

In relation to other differentiation abilities, UCWJ-MSCs are the most studied cell type among various UC compartments and many papers have been published[53,54]. In addition to differentiating into osteocytes, chondrocytes and adipocytes, UCWJ-MSCs can differentiate into cardiomyocytes (with the gene expression of N-cadherin and cardiac troponin I [55]), neurons and glia[20], oligodendrocytes[56] and hepatocytes[57]. Recently, clinical trials have been conducted using UC-MSCs for neurogenic disorders (spinocerebellar ataxia and multiple system atrophy of the cerebellar type)[58] and liver disorders[59,60].

IMMUNOSUPPRESSIVE PROPERTIES OF UC-MSCs

Immunosuppressive effects have now become the most popular property of MSCs for potential clinical use. First, MSCs themselves are weakly immunogenic owing to the lack of HLA-DR and low expression of MHC class I molecules. MSCs have been shown to have immunomodulatory properties *in vitro*[61]. Furthermore, MSCs lack both CD80 and CD86 proteins[36,62], which

are costimulatory molecules inducing T cell activation and survival. The lack of HLA-DR, CD80 and CD86 suggests that MSCs do not elicit acute rejection and are suitable for allogeneic cell-based therapy.

Second, UC-MSCs have immunosuppressive properties *in vitro* and *in vivo*. Many studies have been published about the immunosuppressive effect of UCWJ-MSCs[63], UC lining-MSCs[37,64], HUCPV[65] and whole UC-derived MSCs[66]. The immunosuppressive effect of UC-MSCs is mediated by soluble factors and cell-to-cell contacts. PGE2, galectin-1 and HLA-G5 are released from MSCs and serve as effective factors of immunosuppression[67]. Among these factors, indoleamine 2,3-dioxygenase (IDO) is one of the most relevant because it is inducible by IFN-γ and catalyzes conversion from tryptophan to kynurenine[62,68]. This depletion of tryptophan from the environment can suppress T cell proliferation. UCWJ-MSC-mediated immunosuppression may require preliminary activation by proinflammatory cytokines, such as IFN-γ, with or without TNF-α, IL-1α or IL-1β.

It was recently suggested that the inflammatory environment produced by the upregulation of cytokines such as IFN-γ and TNF-α might alter the biological activity of MSCs from immunosuppression to immunostimulation[68]. In this case, UC-MSCs maynot prevent GVHD *in vivo*. It is known that upon stimulation by activated immune cells or cytokines (priming), MSCs are primed and become functional immunosuppressors. The extent of immunosuppression is greater with UCWJ-MSCs than with BM-MSCs[62]. Polchert *et al*[68] demonstrated that MSCs primed with IFN-γ are effective in a mouse GVHD model despite upregulated MHC class Ⅱ molecules. In order to ensure the effective and safe therapeutic use of UC-MSCs, more *in vivo* experiments need to be conducted because of the many discrepancies with *in vitro* data.

CONCLUSIONS

Compared with the counterparts of other origins, UC-MSCs have attractive advantages as MSCs and as UC-derived cells: (1) a noninvasive collection procedure for autologous or allogeneic use; (2) a lower risk of infection; (3) a low risk of teratoma; (4) multipotency; and (5) low immunogenicity with a good immunosuppressive ability. It is still unclear which compartment in UC is the best for clinical use; nonetheless, the era of the clinical use of UC-MSCs is approaching quickly.

REFERENCES

1 **Gnecchi M**, Melo LG. Bone marrow-derived mesenchymal stem cells: isolation, expansion, characterization, viral transduction, and production of conditioned medium. *Methods Mol Biol* 2009; **482**: 281-294 [PMID: 19089363 DOI: 10.1007/978-1-59745-060-7_18]

2 **Tondreau T**, Meuleman N, Delforge A, Dejeneffe M, Leroy R, Massy M, Mortier C, Bron D, Lagneaux L. Mesenchymal stem cells derived from CD133-positive cells in mobilized peripheral blood and cord blood: proliferation, Oct4 expression, and plasticity. *Stem Cells* 2005; **23**: 1105-1112 [PMID: 15955825 DOI: 10.1634/stemcells.2004-0330]

3 **Bieback K**, Klüter H. Mesenchymal stromal cells from umbilical cord blood. *Curr Stem Cell Res Ther* 2007; **2**: 310-323 [PMID: 18220915]

4 **McElreavey KD**, Irvine AI, Ennis KT, McLean WH. Isolation, culture and characterisation of fibroblast-like cells derived from the Wharton's jelly portion of human umbilical cord. *Biochem Soc Trans* 1991; **19**: 29S [PMID: 1709890]

5 **Ishige I**, Nagamura-Inoue T, Honda MJ, Harnprasopwat R, Kido M, Sugimoto M, Nakauchi H, Tojo A. Comparison of mesenchymal stem cells derived from arterial, venous, and Wharton's jelly explants of human umbilical cord. *Int J Hematol* 2009; **90**: 261-269 [PMID: 19657615 DOI: 10.1007/s12185-009-0377-3]

6 **In 't Anker PS**, Scherjon SA, Kleijburg-van der Keur C, de Groot-Swings GM, Claas FH, Fibbe WE, Kanhai HH. Isolation of mesenchymal stem cells of fetal or maternal origin from human placenta. *Stem Cells* 2004; **22**: 1338-1345 [PMID: 15579651]

7 **Gruber HE**, Deepe R, Hoelscher GL, Ingram JA, Norton HJ, Scannell B, Loeffler BJ, Zinchenko N, Hanley EN, Tapp H. Human adipose-derived mesenchymal stem cells: direction to a phenotype sharing similarities with the disc, gene expression profiling, and coculture with human annulus cells. *Tissue Eng Part A* 2010; **16**: 2843-2860 [PMID: 20408770 DOI: 10.1089/ten.TEA.2009.0709]

8 **Ponnaiyan D**, Bhat KM, Bhat GS. Comparison of immunophenotypes of stem cells from human dental pulp and periodontal ligament. *Int J Immunopathol Pharmacol* 2012; **25**: 127-134 [PMID: 22507325]

9 **Joshi M**, B Patil P, He Z, Holgersson J, Olausson M, Sumitran-Holgersson S. Fetal liver-derived mesenchymal stromal cells augment engraftment of transplanted hepatocytes. *Cytotherapy* 2012; **14**: 657-669 [PMID: 22424216 DOI: 10.3109/14653249.2012.663526]

10 **in 't Anker PS**, Noort WA, Scherjon SA, Kleijburg-van der Keur C, Kruisselbrink AB, van Bezooijen RL, Beekhuizen W, Willemze R, Kanhai HH, Fibbe WE. Mesenchymal stem cells in human second-trimester bone marrow, liver, lung, and spleen exhibit a similar immunophenotype but a heterogeneous multilineage differentiation potential. *Haematologica* 2003; **88**: 845-852 [PMID: 12935972]

11 **Hsieh JY**, Fu YS, Chang SJ, Tsuang YH, Wang HW. Functional module analysis reveals differential osteogenic and stemness potentials in human mesenchymal stem cells from bone marrow and Wharton's jelly of umbilical cord. *Stem Cells Dev* 2010; **19**: 1895-1910 [PMID: 20367285 DOI: 10.1089/scd.2009.0485]

12 **Fong CY**, Chak LL, Biswas A, Tan JH, Gauthaman K, Chan WK, Bongso A. Human Wharton's jelly stem cells have unique transcriptome profiles compared to human embryonic stem cells and other mesenchymal stem cells. *Stem Cell Rev* 2011; **7**: 1-16 [PMID: 20602182 DOI: 10.1007/s12015-010-9166-x]

13 **Troyer DL**, Weiss ML. Wharton's jelly-derived cells are a primitive stromal cell population. *Stem Cells* 2008; **26**: 591-599 [PMID: 18065397 DOI: 10.1634/stemcells.2007-0439]

14 **Gluckman E**, Broxmeyer HA, Auerbach AD, Friedman HS, Douglas GW, Devergie A, Esperou H, Thierry D, Socie G, Lehn P. Hematopoietic reconstitution in a patient with Fanconi's anemia by means of umbilical-cord blood from an HLA-identical sibling. *N Engl J Med* 1989; **321**: 1174-1178 [PMID: 2571931]

15 **Zhang X**, Hirai M, Cantero S, Ciubotariu R, Dobrila L, Hirsh A, Igura K, Satoh H, Yokomi I, Nishimura T, Yamaguchi S,

Yoshimura K, Rubinstein P, Takahashi TA. Isolation and characterization of mesenchymal stem cells from human umbilical cord blood: reevaluation of critical factors for successful isolation and high ability to proliferate and differentiate to chondrocytes as compared to mesenchymal stem cells from bone marrow and adipose tissue. *J Cell Biochem* 2011; **112**: 1206-1218 [PMID: 21312238 DOI: 10.1002/jcb.23042]

16 **Manca MF**, Zwart I, Beo J, Palasingham R, Jen LS, Navarrete R, Girdlestone J, Navarrete CV. Characterization of mesenchymal stromal cells derived from full-term umbilical cord blood. *Cytotherapy* 2008; **10**: 54-68 [PMID: 18202975]

17 **Wexler SA**, Donaldson C, Denning-Kendall P, Rice C, Bradley B, Hows JM. Adult bone marrow is a rich source of human mesenchymal 'stem' cells but umbilical cord and mobilized adult blood are not. *Br J Haematol* 2003; **121**: 368-374 [PMID: 12694261]

18 **Secco M**, Zucconi E, Vieira NM, Fogaça LL, Cerqueira A, Carvalho MD, Jazedje T, Okamoto OK, Muotri AR, Zatz M. Multipotent stem cells from umbilical cord: cord is richer than blood! *Stem Cells* 2008; **26**: 146-150 [PMID: 17932423]

19 **Romanov YA**, Svintsitskaya VA, Smirnov VN. Searching for alternative sources of postnatal human mesenchymal stem cells: candidate MSC-like cells from umbilical cord. *Stem Cells* 2003; **21**: 105-110 [PMID: 12529557]

20 **Mitchell KE**, Weiss ML, Mitchell BM, Martin P, Davis D, Morales L, Helwig B, Beerenstrauch M, Abou-Easa K, Hildreth T, Troyer D, Medicetty S. Matrix cells from Wharton's jelly form neurons and glia. *Stem Cells* 2003; **21**: 50-60 [PMID: 12529551]

21 **Minguell JJ**, Lorino R, Lasala GP. Myocardial implantation of a combination stem cell product by using a transendocardial MYOSTAR injection catheter: A technical assessment. *Acute Card Care* 2011; **13**: 40-42 [PMID: 21323410 DOI: 10.3109/17482941.2010.551134]

22 **Muroi K**, Miyamura K, Ohashi K, Murata M, Eto T, Kobayashi N, Taniguchi S, Imamura M, Ando K, Kato S, Mori T, Teshima T, Mori M, Ozawa K. Unrelated allogeneic bone marrow-derived mesenchymal stem cells for steroid-refractory acute graft-versus-host disease: a phase I/II study. *Int J Hematol* 2013; **98**: 206-213 [PMID: 23860964 DOI: 10.1007/s12185-013-1399-4]

23 **Weng JY**, Du X, Geng SX, Peng YW, Wang Z, Lu ZS, Wu SJ, Luo CW, Guo R, Ling W, Deng CX, Liao PJ, Xiang AP. Mesenchymal stem cell as salvage treatment for refractory chronic GVHD. *Bone Marrow Transplant* 2010; **45**: 1732-1740 [PMID: 20818445 DOI: 10.1038/bmt.2010.195]

24 **Duijvestein M**, Vos AC, Roelofs H, Wildenberg ME, Wendrich BB, Verspaget HW, Kooy-Winkelaar EM, Koning F, Zwaginga JJ, Fidder HH, Verhaar AP, Fibbe WE, van den Brink GR, Hommes DW. Autologous bone marrow-derived mesenchymal stromal cell treatment for refractory luminal Crohn's disease: results of a phase I study. *Gut* 2010; **59**: 1662-1669 [PMID: 20921206 DOI: 10.1136/gut.2010.215152]

25 **Kagami H**, Agata H, Tojo A. Bone marrow stromal cells (bone marrow-derived multipotent mesenchymal stromal cells) for bone tissue engineering: basic science to clinical translation. *Int J Biochem Cell Biol* 2011; **43**: 286-289 [PMID: 21147252 DOI: 10.1016/j.biocel.2010.12.006]

26 **Baksh D**, Yao R, Tuan RS. Comparison of proliferative and multilineage differentiation potential of human mesenchymal stem cells derived from umbilical cord and bone marrow. *Stem Cells* 2007; **25**: 1384-1392 [PMID: 17332507]

27 **Mueller SM**, Glowacki J. Age-related decline in the osteogenic potential of human bone marrow cells cultured in three-dimensional collagen sponges. *J Cell Biochem* 2001; **82**: 583-590 [PMID: 11500936]

28 **Sarugaser R**, Lickorish D, Baksh D, Hosseini MM, Davies JE. Human umbilical cord perivascular (HUCPV) cells: a source of mesenchymal progenitors. *Stem Cells* 2005; **23**: 220-229 [PMID: 15671145]

29 **Marmotti A**, Mattia S, Bruzzone M, Buttiglieri S, Risso A, Bonasia DE, Blonna D, Castoldi F, Rossi R, Zanini C, Ercole E, Defabiani E, Tarella C, Peretti GM. Minced umbilical cord fragments as a source of cells for orthopaedic tissue engineering: an in vitro study. *Stem Cells Int* 2012; **2012**: 326813 [PMID: 22550503 DOI: 10.1155/2012/326813]

30 **Majore I**, Moretti P, Stahl F, Hass R, Kasper C. Growth and differentiation properties of mesenchymal stromal cell populations derived from whole human umbilical cord. *Stem Cell Rev* 2011; **7**: 17-31 [PMID: 20596801 DOI: 10.1007/s12015-010-9165-y]

31 **He H**, Nagamura-Inoue T, Tsunoda H, Yuzawa M, Yamamoto Y, Yorozu P, Agata H, Tojo A. Stage-Specific Embryonic Antigen 4 in Wharton's Jelly-Derived Mesenchymal Stem Cells Is Not a Marker for Proliferation and Multipotency. *Tissue Eng Part A* 2014; **20**: 1314-1324 [PMID: 24279891 DOI: 10.1089/ten.TEA.2013.0333]

32 **Kikuchi-Taura A**, Taguchi A, Kanda T, Inoue T, Kasahara Y, Hirose H, Sato I, Matsuyama T, Nakagomi T, Yamahara K, Stern D, Ogawa H, Soma T. Human umbilical cord provides a significant source of unexpanded mesenchymal stromal cells. *Cytotherapy* 2012; **14**: 441-450 [PMID: 22339605 DOI: 10.3109/14653249.2012.658911]

33 **Salehinejad P**, Alitheen NB, Ali AM, Omar AR, Mohit M, Janzamin E, Samani FS, Torshizi Z, Nematollahi-Mahani SN. Comparison of different methods for the isolation of mesenchymal stem cells from human umbilical cord Wharton's jelly. *In Vitro Cell Dev Biol Anim* 2012; **48**: 75-83 [PMID: 22274909 DOI: 10.1007/s11626-011-9480-x]

34 **Tsagias N**, Koliakos I, Karagiannis V, Eleftheriadou M, Koliakos GG. Isolation of mesenchymal stem cells using the total length of umbilical cord for transplantation purposes. *Transfus Med* 2011; **21**: 253-261 [PMID: 21623971 DOI: 10.1111/j.1365-3148.2011.01076.x]

35 **Bosch J**, Houben AP, Radke TF, Stapelkamp D, Bünemann E, Balan P, Buchheiser A, Liedtke S, Kögler G. Distinct differentiation potential of "MSC" derived from cord blood and umbilical cord: are cord-derived cells true mesenchymal stromal cells? *Stem Cells Dev* 2012; **21**: 1977-1988 [PMID: 22087798 DOI: 10.1089/scd.2011.0414]

36 **Friedman R**, Betancur M, Boissel L, Tuncer H, Cetrulo C, Klingemann H. Umbilical cord mesenchymal stem cells: adjuvants for human cell transplantation. *Biol Blood Marrow Transplant* 2007; **13**: 1477-1486 [PMID: 18022578 DOI: 10.1016/j.bbmt.2007.08.048]

37 **Deuse T**, Stubbendorff M, Tang-Quan K, Phillips N, Kay MA, Eiermann T, Phan TT, Volk HD, Reichenspurner H, Robbins RC, Schrepfer S. Immunogenicity and immunomodulatory properties of umbilical cord lining mesenchymal stem cells. *Cell Transplant* 2011; **20**: 655-667 [PMID: 21054940 DOI: 10.3727/096368910X536473]

38 **Kita K**, Gauglitz GG, Phan TT, Herndon DN, Jeschke MG. Isolation and characterization of mesenchymal stem cells from the sub-amniotic human umbilical cord lining membrane. *Stem Cells Dev* 2010; **19**: 491-502 [PMID: 19635009 DOI: 10.1089/scd.2009.0192]

39 **Zebardast N**, Lickorish D, Davies JE. Human umbilical cord perivascular cells (HUCPVC): A mesenchymal cell source for dermal wound healing. *Organogenesis* 2010; **6**: 197-203 [PMID: 21220956 DOI: 10.4161/org.6.4.12393]

40 **Lü LL**, Song YP, Wei XD, Fang BJ, Zhang YL, Li YF. [Comparative characterization of mesenchymal stem cells from human umbilical cord tissue and bone marrow]. *Zhongguo Shi Yan Xue Ye Xue Zazhi* 2008; **16**: 140-146 [PMID: 18315918]