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Comparative profiling of stemness markers expressed by human umbilical vein endothelial cells and human umbilical cord matrix-derived stem cells

Abdul-Rahman Hayati[1], Adzman-Sellymiah[2], Kien-Hui Chua[3], Raman-Nur Fatin Aqilah[1], Geok-Chin Tan[4], Simat-Siti Fatimah[5], Yun-Hsuen Lim[6], Rahim-Nur Syahrina[1], Mohd-Manzor Nur Fariha*[1]

[1]Department of Medical Science, Faculty of Medicine and Health Sciences, Universiti Sains Islam Malaysia, Kuala Lumpur, Malaysia
[2]Department of Pathology, Hospital Selayang, Selangor, Malaysia
[3]Department of Physiology, Faculty of Medicine, Universiti Kebangsaan Malaysia, Cheras, Kuala Lumpur, Malaysia
[4]Department of Pathology, Faculty of Medicine, Universiti Kebangsaan Malaysia, Cheras, Kuala Lumpur, Malaysia
[5]Biotechnology Research Institute, Universiti Malaysia Sabah, Kota Kinabalu, Sabah, Malaysia
[6]Department of Obstetrics & Gynecology, Faculty of Medicine, Universiti Kebangsaan Malaysia, Cheras, Kuala Lumpur, Malaysia

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ABSTRACT

Mesenchymal stem cells population has been successfully isolated from the matrix and characterised for its stemness properties. Endothelial cells from the vein of the umbilical cord which are usually termed as HUVEC are also of interest to researchers as it can be manipulated *in vitro* to study endothelial cells behaviour and vascular-associated diseases. Immunophenotyping for positive and negative markers of mesenchymal stem cells was performed on HUVEC and compared to the closest stem cells that were isolated from the umbilical cord matrix which is termed as HUCMSC in this study. The expressions of a set of stemness genes were also detected using qPCR. The results showed that HUVEC strongly expressed hematopoietic and mesenchymal stem cells markers (CD31, CD34, CD9, CD44, CD73 and CD90) while HUCMSC possess mesenchymal stem cells properties with strong expression of CD9, CD44, CD73 and CD90. Interestingly, the expression of stemness genes is detected higher in HUVEC compared to HUCMSC with possibilities of having neural properties as evidenced by the high expression of FGF4, FZD9, Nestin and SOX2. Thus, it is suggested that HUVEC exhibited some stem cells characteristics and could be further investigated for possible application in research as well as in future cells therapy.

* Corresponding Author

Name: Dr. M. M. Nur Fariha
Phone: +603-42892491
Email: nurfariha@usim.edu.my

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INTRODUCTION

The human umbilical cord is a valuable discarded tissue as it presents a potential source of 'early' adult stem cells. An umbilical cord-derived stem cell has many advantages such as being available in abundance, painless during collection and of least ethical concern. It also has great potential in stem cell banking as in future, more therapeutic modalities of tissue repair and regeneration will be based on stem cell therapy. Stem cells can be isolated from different components of the umbilical cord. Wang and colleagues (2004) have succeeded in isolating stem cells from the matrix of the umbilical

cord. This was reproduced by Mitchell *et al.,* (2003) and Weiss *et al.,* (2006). Stem cells have also been isolated from the endothelial and subendothelial layer of the umbilical vein (Covas *et al.,* 2003; Romanov *et al.,* 2003; Liu *et al.,* 2007; Kestendjieva *et al.,* 2008) and the amniotic membrane of the umbilical cord (Ilancheran *et al.,* 2007; Simat *et al.,* 2008; Izumi *et al.,* 2009; Mihu *et al.,* 2009; Parolini *et al.,* 2009; Shi *et al.,* 2009; Wei *et al.,* 2009). These cells are considered as adult stem cells although they are not exactly derived from adult tissue. As the stem cells originated from tissues during the development of the embryo, they are more appropriately denoted to as 'nonembryonic' stem cells.

Isolation and characterisation of human umbilical vein endothelial cells (HUVEC) from the umbilical cord tissue was first documented by Jaffe *et al.,* (1973). He reported that monolayer grown HUVEC demonstrated large polygonal cells. Further research on HUVEC was carried out on its property as endothelial cells and also the use of HUVEC as endothelial cells model. The emergence of studies in stem cell therapy has opened a new channel for the application of HUVEC especially in the field of vascular and angiogenesis. Co-culture of HUVEC and stem cells was proven to promote vascular formation (Jiang *et al.,* 2015; Inglis *et al.,* 2016). From these studies, HUVEC is believed to have angiogenic properties and is able to induce undifferentiated stem cells to become endothelial or smooth muscle cells.

Stemness properties of HUVEC have never been discussed before. To be well known as committed and differentiated cells, the question on the stem cell properties is not of importance for HUVEC until our recent coincidental findings that HUVEC expressed CD73 which is a mesenchymal stem cell marker. Therefore, in this current study, we aimed to explore the expression of stem cell-associated markers in HUVEC and compare it to the stem cell properties of umbilical cord matrix stem cells as the closest source. The characterisation of stemness properties includes the quantification of the stemness genes expression level and the expression of stem cell surface markers as previously used in other studies (Wang *et al.,* 2004; Miki *et al.,* 2005; Mihu *et al.,* 2009; Fariha *et al.,* 2011).

MATERIALS AND METHODS

Approval for the study was obtained from the Ethical and Research Committee, Faculty of Medicine, Universiti Kebangsaan Malaysia Medical Centre (UKMMC). The samples were comprised of twelve human umbilical cords, collected from healthy term mother. The cells were isolated from two sources, i.e. the umbilical cord vein and umbilical cord matrix (Wharton's jelly).

Sample Collection and Cell Culture

The umbilical cords were taken after birth and kept within a sterile container containing 40 to 50 ml of cord buffer. The cords were immediately processed or kept at 4°C until processing. Prior to processing the cords, they were examined for any clamp marks or needle punctures, which were later, cut and discarded. The cords were then washed and cleaned with phosphate buffered saline (PBS) (GIBCO, Invitrogen, USA) to remove traces of blood.

Human Umbilical Cord Matrix-Derived Stem Cells (HUCMSC) Isolation and Culture

To isolate stem cells from the umbilical cord matrix, the cord was dissected along its length exposing the umbilical vessels (vein and arteries) and the Wharton's jelly. The umbilical vessels were stripped, leaving the Wharton's jelly and the epithelial layer. The matrix was further separated from the epithelial layer and finely chopped. The tissue was then digested with 0.3% collagenase type I (Worthington Biochemical Corporation, New Jersey) in complete medium with gentle agitation at 37°C for 45 minutes. The suspensions were then centrifuged (Damon/ IEC Division, USA) at 1200 rpm for 10 minutes at room temperature. Red blood cells lysis buffer was later added to wash traces of red blood cells. Finally, the cell pellet was then cultured in complete medium which consists of an equal volume mixture of Ham's F12 medium and Dulbecco's Modified Eagle Medium (DMEM/F-12), 10% fetal bovine serum (FBS), 1% Glutamax, 1% Vitamin C and 1% Antibiotic antimycotic (all from GIBCO Invitrogen, USA). The culture was maintained until passage 3 in a standard culture condition, 5% CO_2 in a 37°C incubator (RS Biotech Galaxy Plus, Scotland, UK). The morphology of the isolated cells is demonstrated in Figure 1a.

HUVEC Isolation and Culture

For HUVEC isolation, the umbilical cord vein was identified by its collapsible lumen and thinner vessel wall as compared to the umbilical artery. The vein was catheterized with a blunt 14 Gauge needle and repeatedly washed with cord buffer to ensure residual blood within the umbilical vein was cleared. Then, both two ends of the umbilical vein were clamped with plastic cable ties, at one end the tie was done loosely. At a loose end, a blunt 14 Gauge needle again was inserted and 0.1% collagenase Type I (GIBCO Invitrogen, USA) was infused. Once the umbilical vein filled up, the needle was withdrawn, and the tie at a loose end tightened up. The vein was left in the shaker incubator (Labline Instruments Inc, Illinois, USA) for 10 minutes at 37°C. Afterwards, one of the cable ties

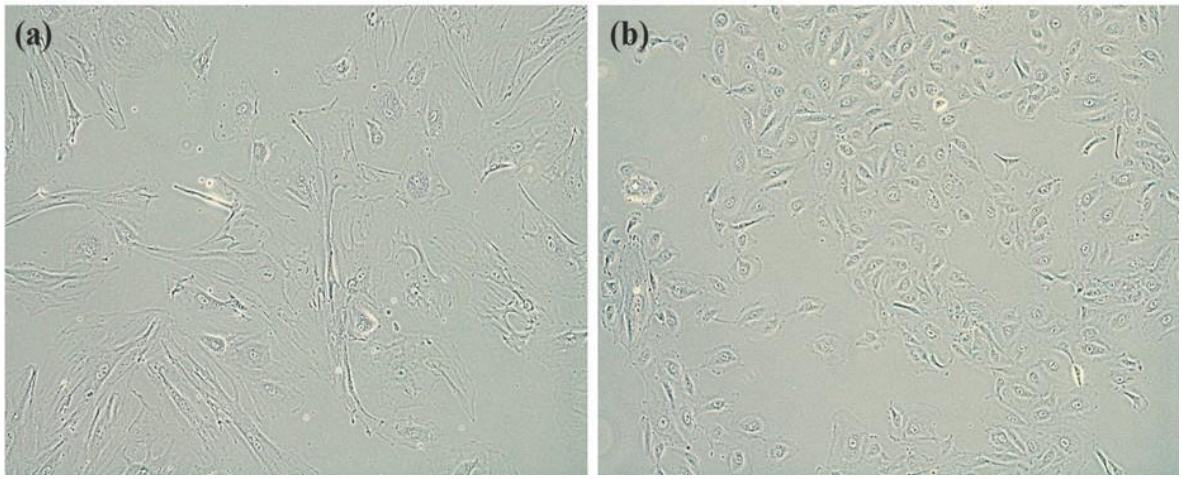

Figure 1: (a) Morphology of HUCMSC and (b) HUVEC at 40x magnification

Table 1: A comparison of the surface marker positivity between HUCMSC and HUVEC

Surface markers	MEAN ± SEM	
	HUCMSC	HUVEC
CD90*	99.92 ± 0.02	23.16 ± 7.77
CD9	82.01 ± 4.15	87.51 ± 3.43
CD45	5.25 ± 2.50	15.49 ± 7.70
CD73*	99.02 ± 0.68	94.82 ± 1.05
CD44	91.03 ± 2.22	77.99 ± 6.08
CD117	5.30 ± 2.89	8.19 ± 2.26
CD34*	3.61 ± 1.97	31.15 ± 7.16
CD31*	3.80 ± 2.33	97.45 ± 0.44
HLA-ABC	98.25 ± 1.26	96.18 ± 0.86
HLA-DR/DP/DQ	4.93 ± 2.38	12.85 ± 6.12

* Indicates significant differences between HUCMSC compared to HUVEC

Table 2: A comparison of the stemness gene expression level of both HUCMSC and HUVEC

Genes	MEAN ± SEM	
	HUCMSC	HUVEC
ABCG2	$1.34 \pm 0.33 \times 10^{-4}$	$2.88 \pm 0.82 \times 10^{-4}$
BST1*	$7.26 \pm 1.20 \times 10^{-3}$	$4.51 \pm 0.51 \times 10^{-3}$
FGF4*	$1.27 \pm 0.37 \times 10^{-7}$	$2.54 \pm 0.61 \times 10^{-6}$
FZD9*	$1.24 \pm 0.17 \times 10^{-5}$	$6.08 \pm 0.61 \times 10^{-5}$
Nanog3*	$1.24 \pm 0.17 \times 10^{-5}$	$8.44 \pm 1.05 \times 10^{-5}$
Nestin*	$2.43 \pm 0.40 \times 10^{-4}$	$3.03 \pm 0.83 \times 10^{-3}$
Oct-3/4	$4.21 \pm 0.85 \times 10^{-5}$	$4.54 \pm 0.65 \times 10^{-5}$
Rex-1	$1.93 \pm 1.50 \times 10^{-6}$	$2.26 \pm 0.71 \times 10^{-6}$
SOX2*	$2.40 \pm 0.32 \times 10^{-5}$	$1.63 \pm 0.36 \times 10^{-4}$

* Indicates significant differences between HUCMSC compared to HUVEC

was cut to drain the collagenase into a 50 ml polypropylene tube (Greiner Bio-one, Germany). An equal amount of complete medium was added and then centrifuged for 10 minutes at 1200 rpm. The cell pellet was resuspended in 10 ml of Endothelial Cell Medium (ECM) supplemented with Endothelial cell growth medium (ECGS) (Science cell, CA). The cells were allowed to adhere in a T25 culture flask (Nunc, Denmark) at 37°C with 5% CO_2 and 95% humidified air, where the culture medium changed every 3 days. When the passage (P)0 cells reached 80% confluence, they were sub-cultured into new T25 flasks with one to four expansion ratios. The cells were detached from the surface of the flask by adding 4 ml of 0.125% trypsin with 1mM EDTA (GIBCO, Invitrogen, USA). The growth medium removed earlier was added back into the flask to deactivate the trypsin activity. All content in the flask was transferred into 50 ml polypropylene tube (Greiner Bio-one, Germany) and pelleted by centrifugation at 1200 rpm for 10 minutes. A fresh medium was added to the cell pellet and vortexed to suspend it. The same culture procedure was conducted until the cells reached passage 3. The morphology of the isolated cells is observed in Figure 1b.

Flow Cytometry Analysis

Flow cytometry was used to identify stem cells' surface markers. It was performed on cells at passage 3. Accutase solution (Innovative Cell Technologies, San Diego) was added to detach the cells and followed by inactivation of accutase using complete medium before it was centrifuged at 1200 rpm for 10 minutes. The cell pellet was then washed using 1X Dulbecco's phosphate-buffered saline (DPBS) (GIBCO, Invitrogen, USA) with 0.5% Bovine Serum Albumin (BSA) (Sigma, USA) and centrifuged for 10 minutes at 1200 rpm. The washed cells were suspended once again in 1X DPBS with 0.5% BSA and filtered through 70 um nylon membrane. Cell count was done using haemocytometer where 10 µl of Trypan blue (Becton Dickinson, Belgium) in 90 µl of cell suspension was used to identify dead cells. A minimum number of 100,000 cells were collected for each reaction tube incubated with single or combined FITC and PE-conjugated antibodies; CD90/FITC and CD9/PE, CD45/FITC and CD73/PE, CD44/FITC and CD117/PE, CD34/FITC and CD31/PE, HLA-DR DP DQ/FITC, HLA-ABC/FITC (BD Bioscience Pharmingen, San Jose, US). For each antibody set, 10,000 events were acquired using CELLQuestPro acquisition software on a FACSCalibur flow cytometer (Becton, Dickinson and Company, Franklin Lakes, NJ, http://www.bd.com).

RNA Extraction and Quantitative Polymerase Chain Reaction (qPCR)

Cultured cells from passage 3 were harvested for RNA extraction. Total RNA was extracted using TRI-reagent (MRC), and a sample of 100 to 200 ng was reversed transcribed with reverse transcriptase (RT) (Invitrogen, USA) for 30 minutes at 50°C in the presence of oligo-dT primer (Invitrogen). qPCR was carried out using specific primer sequences that were published in a previous study (Fariha *et al.,* 2011). The reaction was performed with 5 uM of each primer and SYBR Green (Biorad, USA) as an indicator in a Bio-Rad iCycler (Biorad, USA) instrument for 40 cycles, 95°C (10 seconds) and 61°C (30 seconds). To ensure specificity of the reactions, the melting profile of each primer set was verified, and the qPCR product was further confirmed by electrophoresis on 2% agarose gel (ICN Biomedicals). The genes that were selected for qPCR are as follows; ATP binding cassette subfamily G member 2 (ABCG2), Bone marrow stromal cell antigen 1 (BST1), Fibroblast growth factor 4 (FGF4), Frizzled class receptor 9 (FZD9), Nanog3, Nestin, Octamer-binding protein ¾ (Oct-3/4), Reduced expression 1 (Rex-1), SRY-related HMG-box 2 (SOX2), and Telomerase reverse transcriptase (TERT). The expression level of each gene was then normalized to Glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

Statistical Analysis

Mann-Whitney U test was used to compare the mean of each variable expressed by cells from each group. The differences observed in Table 1 and Table 2 were tested for statistical significance with SPSS 12.0. For flow cytometry, the mean rank of each marker expressed by the stem cells from HUCMSC and HUVEC were compared and analysed. Similarly, for qPCR, the mean of each gene expression of interest was also evaluated.

RESULTS AND DISCUSSION

Expression of Stem Cells' Surface Markers on HUCMSC and HUVEC

The flow cytometry results of HUCMSC and HUVEC are presented in Table 1. The representative flow cytometry histograms of surface markers expressed by the cells were presented in Figure 2 and 3. It was regarded as positive if at least 3% of the cells showed a positive reaction towards each antibody. The positive expressions were further categorised as follows: 3-20% = weak; 21-50% = medium; 51-80% = medium high/ strong and >80% = high/strong (Sundberg *et al.,* 2009). The expression profiles were similar to that of mesenchymal stem cells (MSC), being strong in CD90, CD73, HLA-ABC, CD44 and CD9; and weak in CD117, CD45, HLA-DR/DP/DQ, CD31 and CD34.

HUVEC strongly expressed CD31 (one of the vascular markers), HLA-ABC, CD73 and CD9. Weak expressions were observed with CD45, HLA DR/DP/DQ and CD117. With the exception of HLA-ABC, more than 20% of the cell population expressed CD73, CD9, CD44 and CD90 (mesenchymal markers). The other haematopoietic markers, CD34 and CD45, were also expressed but were relatively weaker. Hence, we observed a mixed profile of endothelial cells, mesenchymal stem cells and haematopoietic stem cells.

HUCMSC expressed more of CD90, CD73, CD44 and HLA-ABC whilst HUVEC expressed more of CD9, CD45, CD117, CD34, CD31 and HLA-DR/DP/DQ. Hence, HUCMSC have stronger mesenchymal stem cells properties while HUVEC have stronger endothelial cells and haematopoietic stem cell properties. As for the immunological properties, although HLA-DR/DP/DQ was expressed more in HUVEC, its expression in both HUCMSC and HUVEC were very much lower than the expression of HLA-ABC. The statistical analysis showed that the HUCMSC expressed more CD90 and CD73 (mesenchymal markers) as compared to HUVEC ($p<0.05$). On the other hand, HUVEC have strong expression of

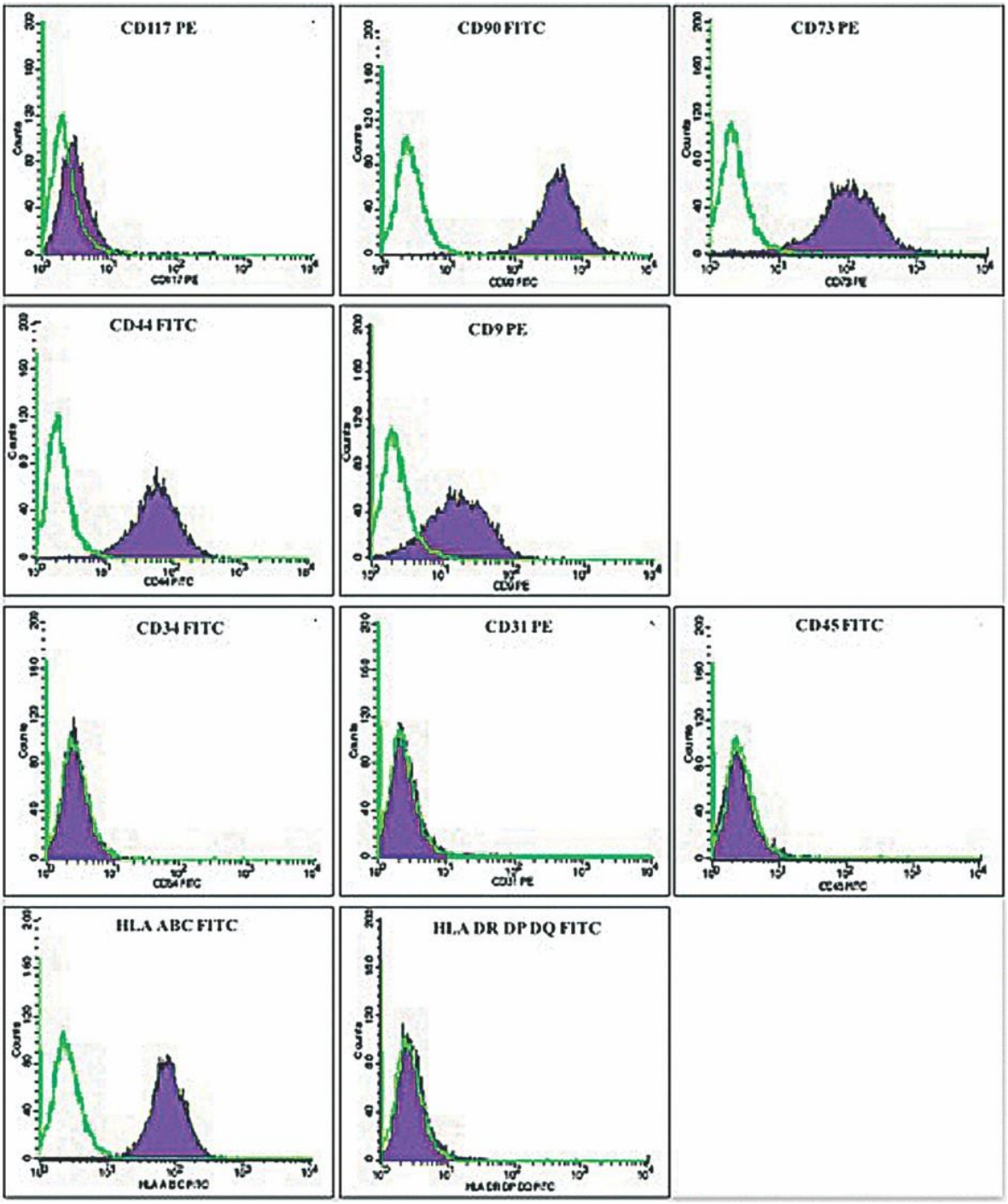

Figure 2: Representative flow cytometry histograms showing the surface marker profile of passage 3 HUCMSC

CD31 and CD34 (vascular markers) as compared to HUCMSC; both had significant values ($p<0.05$).

It can be inferred from our study that HUVEC possess both mesenchymal and haematopoietic profiles and HUCMSC have mesenchymal stem cell characters. The latter is similar to the findings by Weiss *et al.,* (2008), where stem cells isolated from the umbilical cord matrix were a member of the mesenchymal stem cells family. However, HUVEC have a mixed profile of both mesenchymal and hematopoietic stem cell properties. This finding is partly in contrast to that of Covas *et al.,* (2003) and Romanov *et al.,* (2003) where HUVEC had mesenchymal-like properties which were then induced to differentiate into adipogenic and osteogenic cells. Covas *et al.* showed that cells derived from the endothelium and subendothelium of the umbilical vein were composed of adherent cells with fibroblastoid morphology. Romanov *et al.* also cultured the endothelial cells of the umbilical vein in a bone marrow-derived mesenchymal stem cells environment. They found that the initial culture had a typical endothelial morphology following which there

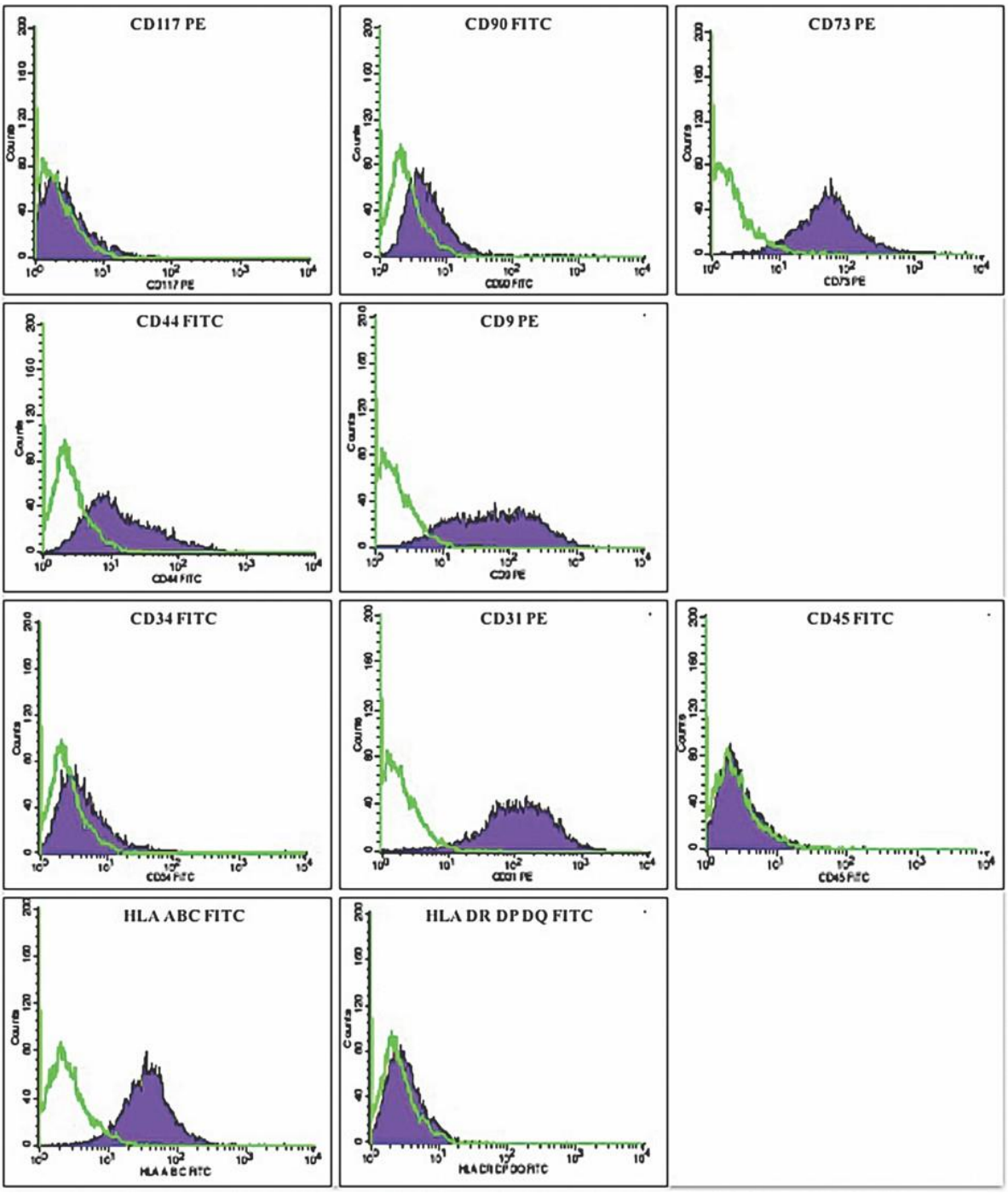

Figure 3: Representative flow cytometry histograms showing the surface marker profile of passage 3 HUCMSC

was a growth of fibroblast-like cells in between the endothelial cells. The fibroblast-like cells were negative while the endothelial-like cells were positive for von Willebrand factor and PECAM-1. They have proven that the isolated cells were endothelial but being in a medium that favours mesenchymal growth, thus mesenchymal trans-differentiation occurred. This observation explains the HUVEC's biphenotypic expressions of a hematopoietic and mesenchymal profile in this study. However, further differentiation analysis was not done on these isolated cells and should be explored.

As with Covas *et al.,* (2003), Romanov *et al.,* (2003), Liu *et al.,* (2007) and Conconi *et al.,* (2006), we reported similar immunological properties of positive HLA-ABC expression with weak or negative MHC Class II (HLA-DP/DQ/DR) in both groups.

Stemness Genes Expression of HUCMSC and HUVEC

The genes expressed by HUCMSC and HUVEC were recorded in Table 2. From the table, all the genes were expressed except for TERT gene. The top three genes expressed were BST-1, Nestin and

ABCG2. The least three genes were Nanog3, Rex-1 and FGF-4. With the exception of BST1 gene, all of the other genes were quantitatively expressed more in HUVEC as compared to HUCMSC. The expression of FGF4, FZD9, Nanog3 and Nestin in HUVEC were at least five times to that observed in HUCMSC. Statistical analysis on the gene expression showed that there were significantly higher expressions of FGF4, FZD9, Nanog3, Nestin and Sox2 in HUVEC as compared to HUCMSC ($p<0.05$). As for HUCMSC, significantly higher expression of BST-1 was also detected when compared to HUVEC ($p<0.05$). The differences of other genes in these two groups were not statistically significant. The expressions of ABCG2, Oct-3/4 and Rex-1 genes were almost similar in both HUCMSC and HUVEC. TERT was not expressed in both groups. There were statistically significant differences between HUCMSC's and HUVEC's expressions of six genes (BST1, FGF4, FZD9, Nanog3, Nestin and SOX2). Interestingly, HUVEC expressed more of FGF4, FZD9, Nanog3, Nestin and SOX2 genes compared to HUCMSC and only BST-1 gene was expressed more in HUCMSC.

When looking at the threshold cycle (C_t) value for the pluripotent genes, the C_t values were very high compared to the C_t value of the housekeeping genes and resulted in low expressions of pluripotent markers (FGF4, FZD9, Nanog3, Oct-3/4, Rex-1 and SOX2) in both groups. Expression of pluripotent markers is usually detected high in undifferentiated cells and embryonic stem cells as they are the most pluripotent type of stem cells. An earlier study by Takeuchi and co-workers (1993) found that the stromal cells of the Wharton's Jelly were myofibroblast rather than the typical fibroblast. Specifically, these fibre-producing cells have contractile properties of smooth muscle cells. Further research by Nanaev and colleagues (1997) revealed that there are three different types of fibroblast with different properties depending on the location they exist within the cord. The most immature, undifferentiated cells possessing mesenchymal stem cells properties are located close to the amniotic surface. The stromal cells with cytoskeletal features are found in increasing distance from the amniotic surface, while the differentiated myofibroblasts are seen close to the umbilical vessels. This suggests that our isolated population of undifferentiated mesenchymal cells probably is a mixture of cells consisting of differentiated stromal cells and myofibroblasts. Hence this could have contributed to the very low expressions of the pluripotent markers. However, further confirmation of the pluripotent properties of different sites of the Wharton's Jelly is needed. Furthermore, in our study, the level of Oct-3/4 expressed by the stem cells from both are possibly suboptimal to maintain the cells' pluripotency and are evidently reflected by the relatively low expressions of other pluripotent markers. Niwa *et al.,* (2000) made a point in their study regarding the quantitative expression of Oct-3/4 that defined the status of embryonic stem cells. They found that a critical amount of Oct-3/4 was needed to maintain the cells' pluripotency. An increment of less than two-fold had caused the embryonic cells to differentiate and the repression of Oct-3/4 had induced loss of pluripotency and dedifferentiation.

From the results of gene expressions demonstrated in Table 2, all genes except for BST1 were expressed significantly more in HUVEC. As described earlier, HUVEC in this study might have differentiated to mesenchymal phenotype when cultured in non-endothelial cells medium. A study by Ho *et al.,* (2010) reported that HUVEC expressed high levels of endogenous Kruppel-like factor 4 (KLF4) which suggests ease of reprogramming or differentiating. This could be a reason for trans-differentiation of HUVEC to mesenchymal cells when cultured in normal medium for stem cells. Surprisingly, when correlated to the surface marker profile, more than 90% of this HUVEC population expressed CD31, a differential marker for endothelial cells. The explanation for this could be because of co-expression of CD31 in the newly transformed HUVEC as reported by Zeisberg *et al.,* (2008). In the study, 30 to 50% of the endothelial cells which have undergone the transition to fibroblast cells co-expressed CD31 and fibroblast markers. The gene expression profile also demonstrated high expressions of SOX2 and Nestin in HUVEC as compared to HUCMSC. Besides been known as pluripotent markers, both genes also serve as neural stem cell markers. Expressions of SOX2 and Nestin are important to maintain the neural stem cells properties including proliferation/survival, self-renewal and neurogenesis (Favaro *et al.,* 2009; Park *et al.,* 2010). In addition, Tanaka *et al.,* (2004) revealed that SOX2 also regulates Nestin expressions. Reduction of SOX2 subsequently results in a reduction of Nestin. This positive regulation of SOX2 towards Nestin probably was captured too in our HUVEC and HUCMSC gene expression profiles where both genes expressions were distinctly high compared to other genes. Furthermore, if compared between cells, HUVEC might express more neuronal properties as evidenced by its high neural gene expression. This hidden neuronal property of HUVEC might contribute to the successful generation of HUVEC-derived neurons and astrocytes (Haile *et al.,* 2015). However, a confirmatory of neuronal properties by induction into neuronal differentiation was not done. From our previous work on chorion-derived stem cells, we observed an upregulation of SOX2 and Nestin in these cells

after angiogenic induction (Abdul *et al.,* 2008). HUVEC are known to be the key player of angiogenesis *in vitro*. Therefore, it is suggested that both SOX2 and Nestin could play certain roles not only in neurogenesis but also in angiogenesis despite their primary role in maintaining stemness characteristics of stem cells.

CONCLUSION

In conclusion, we observed that HUVEC possess hematopoietic and mesenchymal stem cells properties (CD31+, CD34+, CD9+, CD44+, CD73+, CD90+) while HUCMSC possess mesenchymal stem cells properties (strong expression of CD9+, CD44+, CD73+ and CD90+). HUVEC seem to express higher stemness gene than HUCMSC and probably have neural properties as evidenced by the high expression of FGF4, FZD9, Nestin and SOX2. Further subtyping of HUVEC are suggested to confirm its character and commitment into other types of differentiated cells, for example, HUVEC that possess neural markers are further induced into neurogenic differentiation. Thus, hidden properties of HUVEC such as its stemness and neurogenic characteristics should also be further evaluated and confirmed.

Expectantly this study will provide additional knowledge of the properties of cells from a different component of the umbilical cord, a tissue that is often discarded. Umbilical cord promises an easily accessible and cheap source of stem cell, and its potential needs to be further investigated.

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