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Brief Report: The Potential Role of Epigenetics on Multipotent Cell Differentiation Capacity of Mesenchymal Stromal Cells

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ABSTRACT

Human umbilical cord perivascular cells (HUCPVCs) are a readily available source of mesenchymal stromal cells (MSCs) for cell therapy. We were interested in understanding how differences from human bone marrow (BM)-derived MSCs might yield insights into MSC biology. We found that HUCPVCs exhibited increased telomerase activity and longer telomeres compared with BM-MSCs. We also observed enhanced expression of the pluripotency factors OCT4, SOX2, and NANOG in HUCPVCs. The methylation of *OCT4* and *NANOG* promoters was similar in both cell types, indicating that differences in the expression of pluripotency factors between the MSCs were not associated with epigenetic changes. MSC methylation at these loci is greater than reported for embryonic stem cells but less than in dermal fibroblasts, suggesting that multipotentiality of MSCs is epigenetically restricted. These results are consistent with the notion that the MSC population (whether BM- or HUCPV-derived) exhibits higher proliferative capacity and contains more progenitor cells than do dermal fibroblasts. STEM CELLS *2013;31:215–220*

Disclosure of potential conflicts of interest is found at the end of this article.

INTRODUCTION

Human umbilical cord perivascular cells (HUCPVCs), derived from the region surrounding the blood vessels within the human umbilical cord, are an attractive and readily available alternative source of mesenchymal stromal cells (MSCs) for cell therapy [1] and may overcome some of the limitations of bone marrow (BM)-derived MSCs, such as a decline in function with donor age [2, 3]. Furthermore, HUCPVCs have a higher clonogenic frequency than BM-MSCs and contain self-renewing stem cells with multilineage differentiation potential in vitro and in vivo [4]. The molecular characteristics of HUCPVCs, however, have not been previously studied. We hypothesized that the enhanced properties of HUCPVCs, including higher stem cell frequency and greater multipotency, compared with BM-MSCs, result from their neonatal tissue origin.

There are three main transcription factors, OCT4, SOX2, and NANOG, that govern embryonic stem cells' (ESCs) self-renewal and pluripotency [5]. These factors are also expressed in BM-MSCs, suggesting a similar regulatory role although their levels are significantly lower than in ESCs [6]. Whether they mediate MSC multipotency, however, is still unknown. In this study, we characterized different molecular aspects of HUCPVCs compared to BM-MSCs including telomerase activity, telomeres length, expression of pluripotency factors, and OCT4 and NANOG promoter methylation patterns to gain insights into MSC biology.

METHODS

Full methods and any associated references are available as Supplementary Information in the online version of the paper.

MSC Culture, Proliferation Assay, and Osteogenic Differentiation

Umbilical cords were donated by full-term caesarian patients, and BM-MSCs were obtained from healthy young adult volunteers (in their 20s and 30s) after written informed consent according to a protocol approved by the UHN Research Ethics Board. HUCPVCs and BM-MSCs were grown until passage 4. Population doubling time was calculated from carboxyfluorescein diacetate succinimidyl ester proliferation assay time-series data. Osteogenic differentiation was determined by Alizarin Red S staining and alkaline phosphatase activity assay.

Quantitative Telomerase Repeat Amplification Protocol Assay and Telomere Length Assay

Telomerase activity was assessed in cell extracts using a real-time polymerase chain reaction (PCR) assay that measures the

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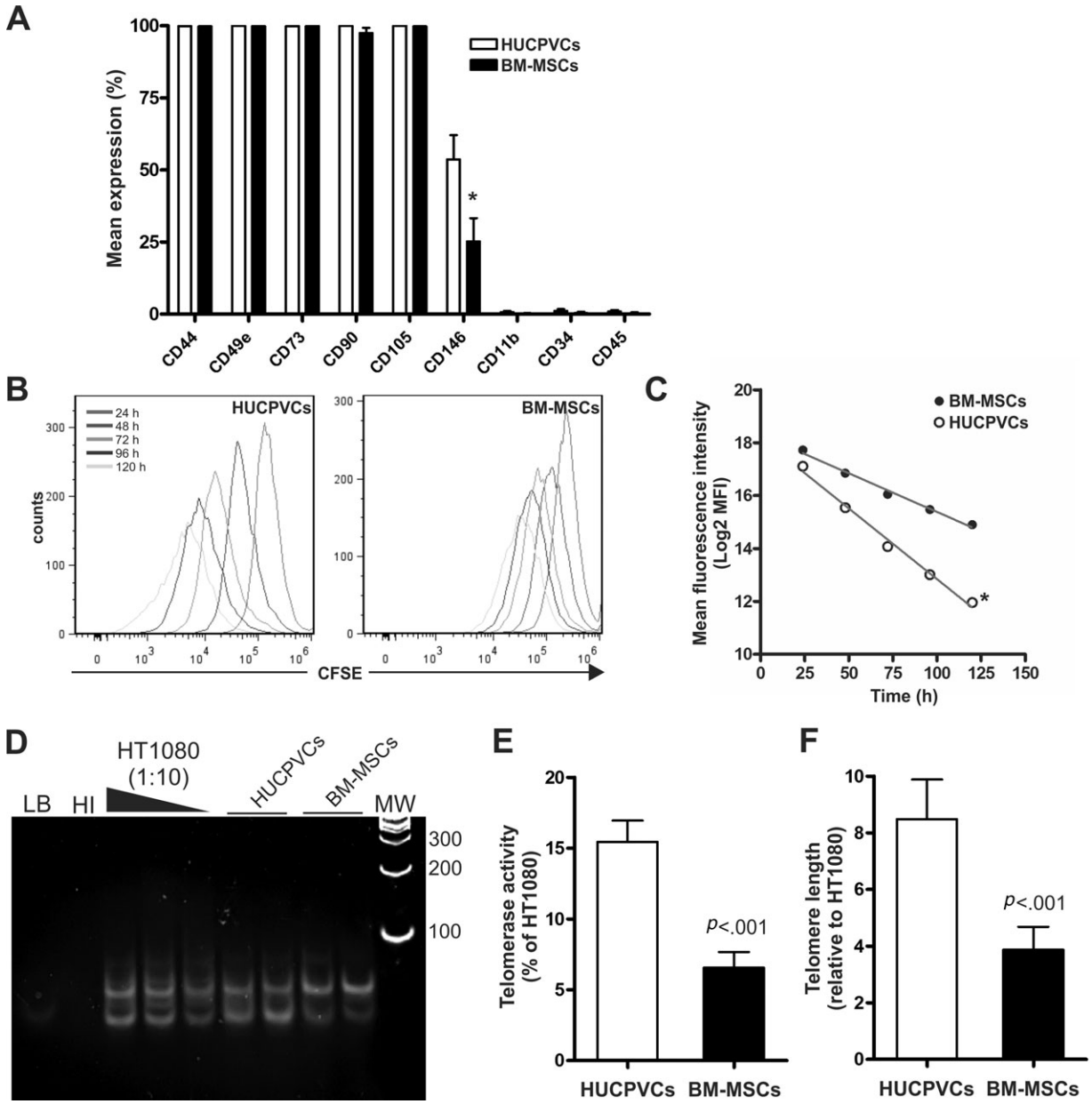

Figure 1. HUCPVCs exhibit a higher proliferation rate, telomerase activity, and longer telomeres compared with BM-MSCs. **(A):** Surface marker expression levels in HUCPVCs and BM-MSCs analyzed by flow cytometry. Human MSCs highly express (>98%) the stromal determinants CD44, CD49e, CD73, CD90, and CD105 and are negative (<1%) for monocyte/macrophage (CD11b), endothelial (CD34), and hematopoietic (CD45) markers. HUCPVCs exhibit a significantly higher expression of the pericyte marker CD146 compared with BM-MSCs. Data represent mean ± S.D. ($n = 9$). *, $p < .0001$ between groups derived from unpaired t test. **(B):** In vitro proliferation assay using CFSE-labeled HUCPVCs and BM-MSCs. The histograms of the CFSE intensity distribution were obtained by flow cytometry at different times (24–120 hours). The decay of CFSE intensity is proportional to the cell proliferation rate. Figure representative of three independent experiments. **(C):** Quantitative analysis of proliferation data. Cell population doubling time was calculated as 1/slope from a Log_2(MFI) versus time plot of CFSE time-course data. The MFI for each time point was determined using a histogram analysis. Data represent mean ± S.D. ($n = 9$). *, Slopes are statistically different with $p < .0001$ derived from t test. **(D):** Analysis of telomerase activity using quantitative telomerase repeat amplification protocol assay (Q-TRAP). Real-time polymerase chain reaction (PCR) products were run on a 10% nondenaturing PAGE to confirm the presence/absence of telomerase products. Positive telomerase activity is evidenced as an incremental TRAP ladder of telomerase products. Serial dilutions (1:10) of telomerase-positive HT1080 whole-cell lysates result in a decreased intensity of the TRAP ladder. A LB and a HI control were included to confirm that the activity is due to the telomerase enzyme and not to nonspecific amplification. HUCPVCs and BM-MSCs are shown to be positive for telomerase activity. **(E):** Relative amounts of telomerase activity assayed by Q-TRAP. Results are expressed as percentage (±S.D.) relative to HT1080 activity ($n = 9$ per group). p values derived from unpaired t test. **(F):** Telomere length measurement by real-time quantitative PCR. Relative telomere length was determined using mean C_t values to calculate the telomere/GAPDH gene ratio, and results are presented taking the telomere length of HT1080 as one ($n = 9$ per group). p values derived from unpaired t test. Abbreviations: BM-MSCs, bone marrow-derived mesenchymal stromal cells; CFSE, carboxyfluorescein diacetate succinimidyl ester; HUCPVCs, human umbilical cord perivascular cells; HI, heat inactivated control; LB, lysis buffer; MW, molecular weight marker; MFI, mean fluorescence intensity.

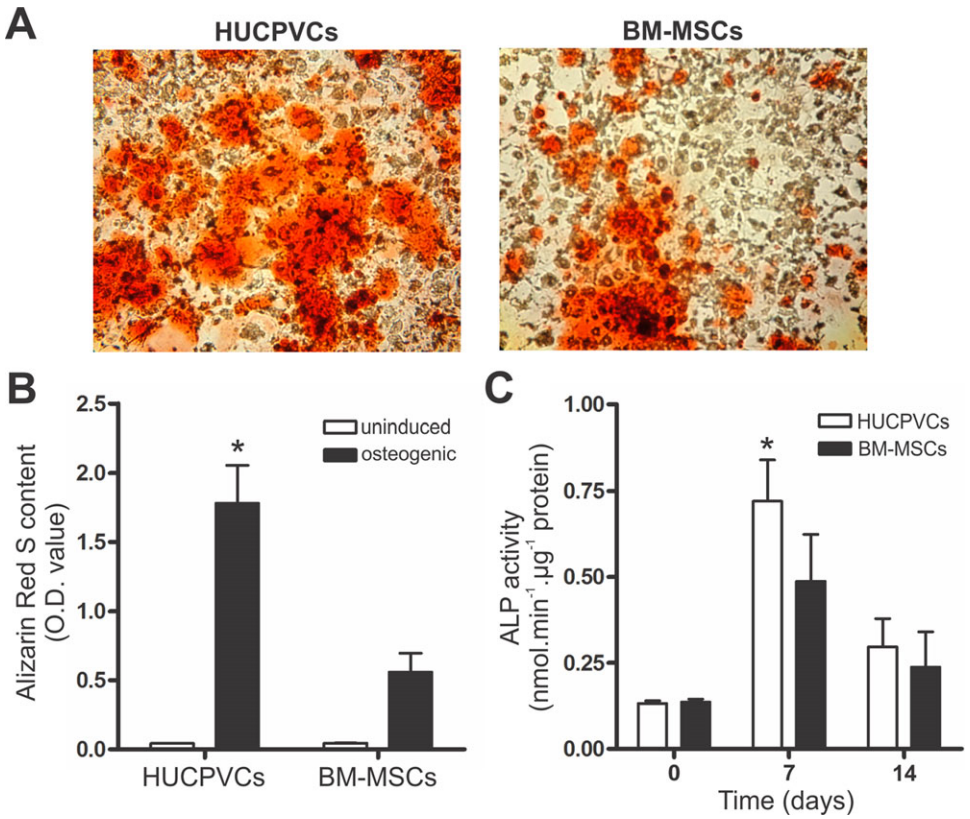

Figure 2. HUCPVCs show enhanced osteogenic differentiation potential compared with BM-MSCs. **(A):** Alizarin Red S staining was performed 20 days after osteogenic induction to exhibit calcium deposition in the extracellular matrix. Representative images of HUCPVCs and BM-MSCs ($n = 6$ per group). Original magnification ×200. **(B):** Calcium mineral content was quantified by dye extraction and spectrophotometry at 570 nm. Data represent mean ± S.D. ($n = 6$). *, $p < .0001$ between groups derived from unpaired t test. **(C):** The kinetics of ALP induction was assessed at the time points of differentiation indicated. ALP activity was assayed with p-nitrophenyl phosphate as a substrate and normalized against total protein concentration. Data represent mean ± S.D. ($n = 5$). *, $p < .05$ between groups derived from unpaired t test. Abbreviations: ALP, alkaline phosphatase; BM-MSCs, bone marrow-derived mesenchymal stromal cells; HUCPVCs, human umbilical cord perivascular cells.

ability of telomerase to extend an exogenous primer [7]. Relative telomere length was measured from genomic DNA samples by a real-time quantitative PCR method described by O'Callaghan et al. [8].

Expression of Pluripotency Factors in MSCs

Quantitative reverse transcriptase PCR (RT-PCR) for *OCT4*, *SOX2*, and *NANOG* was performed as previously described [9], and data were analyzed by the relative quantification ($2^{-\Delta\Delta C_t}$) method [10]. For flow cytometry analysis, cells were fixed, permeabilized, and stained using the following antibodies (Abcam, Cambridge, U.K., http://www.abcam.com): rabbit anti-OCT4 (1:200), rabbit anti-SOX2 (1:200), and rabbit anti-NANOG (1:100). Cytospin slides were also prepared from cultured HUCPVCs and BM-MSCs, and cells were stained for OCT4 (1:300), SOX2 (1:300), and NANOG (1:100) using the primary antibodies described above.

Bisulfite Genomic Sequencing

Bisulfite genomic DNA sample treatment and processing were performed simultaneously for both cell types to analyze the *OCT4* and *NANOG* promoters using previously designed primers [9]. Bisulfite conversion efficiency of non-CpG cytosines was higher than 95% for all individual clones for each sample.

Statistical Analysis

Continuous variables are expressed as mean ± S.D. The unpaired Student t test was used to evaluate statistical significance between HUCPVCs and BM-MSCs. A probability value <.05 was considered statistically significant.

Results and Discussion

First, we determined the immunophenotypic characteristics and proliferation potential of both MSC populations (Fig. 1A–1C; Supporting Information Figs. S1, S2). The proliferation rate of HUCPVCs was significantly higher than for BM-MSCs (population doubling time: 23.6 vs. 44.4 hours, respectively; $p < .001$). Of note, HUCPVCs contain higher levels of $CD146^+$ cells compared with BM-MSCs. CD146 is an endothelial and progenitor cell marker recently described as a marker of pericytes, which identifies cell-renewing and multipotent clonogenic stromal progenitors [11]. Single cell-derived clonal analysis also demonstrated that HUCPVCs are capable of self-renewal and multilineage differentiation, two essential properties of progenitor cells [4].

Growing evidence supports that telomerase activity may affect processes linked to optimum stem-cell function, such as cell replication and differentiation [12]. In fact, a minimum expression of telomerase is required for MSC differentiation into adipocytes and chondrocytes [13], while bone formation is significantly enhanced in telomerase-overexpressing MSCs [14]. Moreover, MSCs undergoing reprogramming showed decreased telomerase activity, indicating cell maturation and terminal differentiation [6]. Interestingly, we found that

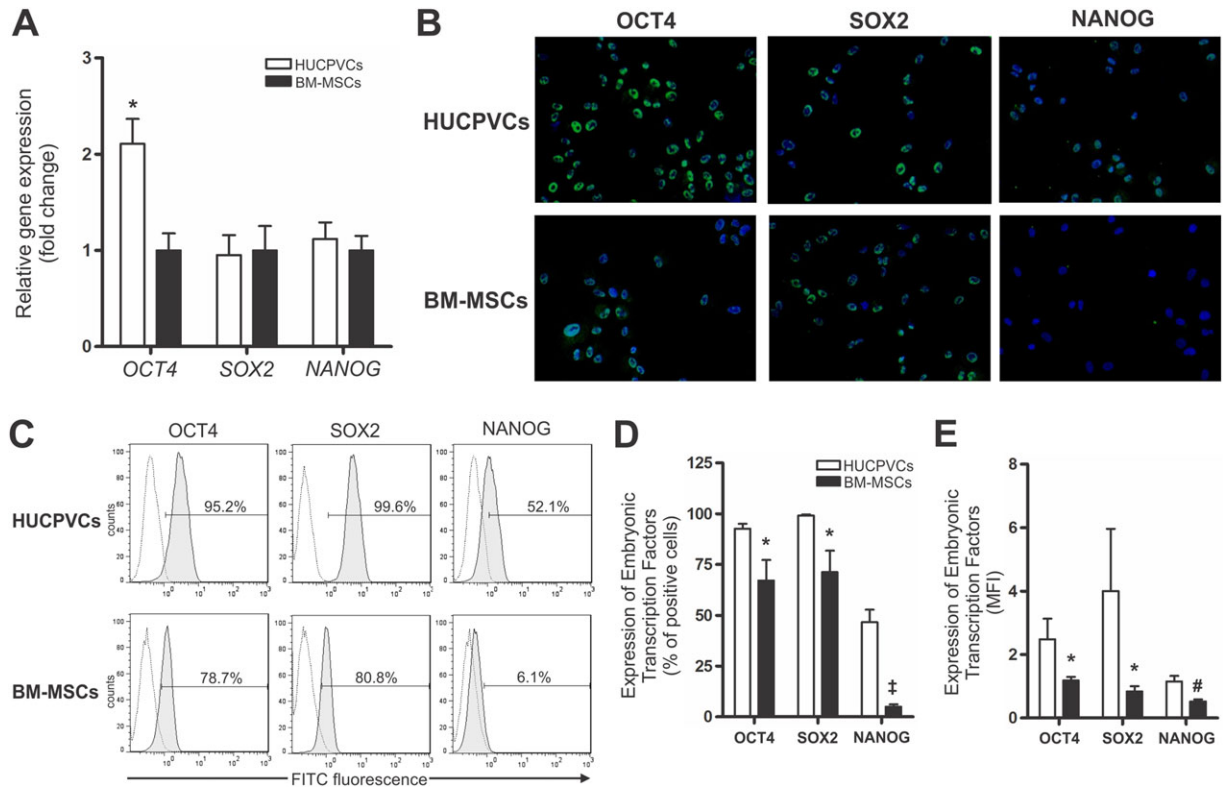

Figure 3. Expression of pluripotency factors in HUCPVCs and BM-MSCs. **(A):** Quantitative real-time polymerase chain reaction (PCR) assay for expression of *OCT4*, *SOX2*, *NANOG*. Results were normalized against a set of reference genes and plotted relative to the expression level of BM-MSCs ($n = 9$ per group). *, $p < .0001$ between groups derived from unpaired t test. **(B):** Expression of embryonic markers (all green) in HUCPVCs and BM-MSCs by immunocytochemistry. Cell nuclei were stained with 4′,6-diamidino-2-phenylindole (blue). Original magnification: ×100. **(C):** Flow cytometry analysis of OCT4, SOX2, and NANOG expression in HUCPVCs and BM-MSCs. Solid gray histograms represent cells stained with FITC-labeled antibodies; isotype-matched controls are overlaid in a dotted black line. Graphs are representative of three independent experiments. **(D):** Percentage of positive cells for the expression of embryonic markers assayed by flow cytometry ($n = 9$ per group). *, $p < .05$ and ‡, $p < .001$ between groups derived from unpaired t test. **(E):** Average amount of pluripotency factors in each cell population represented by MFI ($n = 9$ per group). *, $p < .05$ and #, $p < .01$ between groups derived from unpaired t test. Abbreviations: BM-MSCs, bone marrow-derived mesenchymal stromal cells; HUCPVCs, human umbilical cord perivascular cells; FITC, fluorescein isothiocyanate; MFI, mean fluorescence intensity.

HUCPVCs exhibited 2.4-fold higher telomerase activity (Fig. 1D, 1E; Supporting Information Fig. S3) and significantly longer telomeres (2.2-fold difference) than BM-MSCs (Fig. 1F; Supporting Information Fig. S4). Telomerase activity significantly decreased (<1%) in both cell types after osteogenic differentiation. It is known that telomerase expression is insufficient to fully maintain telomeres in tissue stem cells, a mechanism believed to be responsible for the decreased function of MSCs with age [15]. In this regard, HUCPVCs showed a higher differentiation capacity toward the osteogenic lineage compared with BM-MSCs (Fig. 2). These data are in agreement with previous studies demonstrating enhanced trilineage differentiation capacity of HUCPVCs [4, 16]. Human MSCs derived from embryonic and fetal sources have also exhibited the ability to enhance cardiomyogenesis compared with their adult counterparts [17]. Moreover, several reports have recently demonstrated the regenerative potential of HUCPVCs in different animal models [18–20]. Taken together, these data support the use of HUCPVCs as a more homogeneous source of stromal progenitors and potentially more useful for cell *replacement* therapy, or more likely, in better stimulating the endogenous recovery of injured tissue. Although the immune privileged status of MSCs enables their use in allogeneic cell therapies [21], BM-MSCs are particularly useful for autologous transplantation as HUCPVC banking has not been fully developed.

We next asked whether the enhanced proliferative and multipotent properties of HUCPVCs were related to increased expression of pluripotency factors. We found that *OCT4* was upregulated in HUCPVCs compared with BM-MSCs, however, there was no difference in *SOX2* and *NANOG* gene expression (Fig. 3A). Protein expression was significantly higher in HUCPVCs (Fig. 3B, 3C) not only with respect to the frequency of positive cells (Fig. 3D) but also in relative protein content per cell (Fig. 3E) as assessed by flow cytometry. It is known that post-translational modifications can also regulate OCT4 levels, hence pluripotency [22]. Moreover, *OCT4* knockdown in human BM-MSCs also decreases *SOX2* and *NANOG*, affecting genes associated with differentiation and stem cell maintenance [6]. These data suggest that pluripotency factors may mediate greater clonogenicity and multipotent potential of HUCPVCs compared with BM-MSCs. Current evidence suggests that DNA methylation is the major epigenetic mechanism regulating the expression of stemness genes [9, 23]. While we found reduced *NANOG* promoter methylation in HUCPVCs versus BM-MSCs (16% vs. 27%, $p = .026$), both cells types showed comparable methylation of the *OCT4* promoter (51% vs. 48%, $p = .590$). These results indicate that differences in expression of pluripotency factors between the MSCs were not associated with important epigenetic changes (Fig. 4). Similarly, a recent report found that DNA methylation profiles of lineage-specification genes are

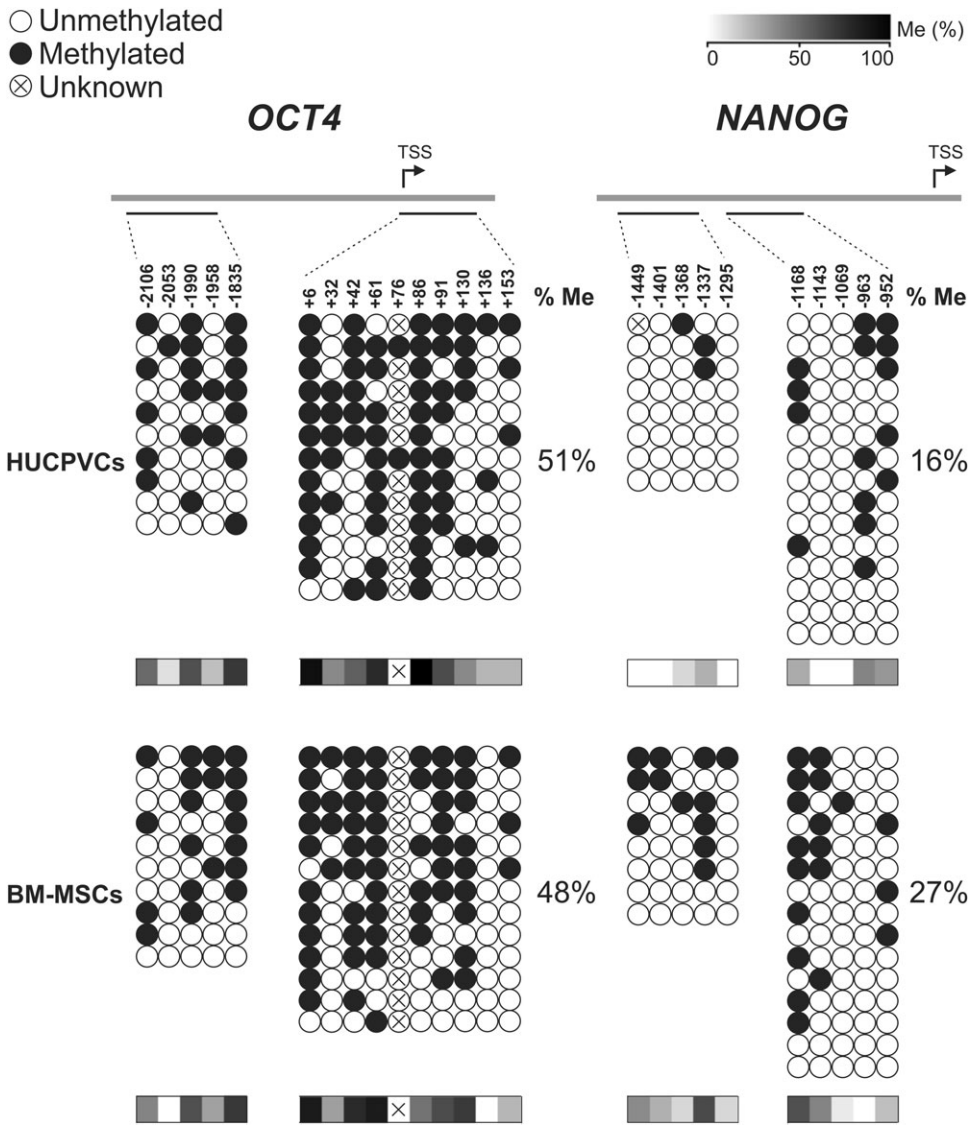

Figure 4. Differences in the expression of pluripotency factors between HUCPVCs and BM-MSCs are not associated with changes in *OCT4* and *NANOG* promoter methylation. Bisulfite sequencing analysis of the *OCT4* (regions 4 and 9) and *NANOG* (regions 1 and 2) promoters in HUCPVCs and BM-MSCs. Open and closed circles indicate unmethylated and methylated CpGs. The values above each column indicate the CpG position relative to the TSS, and a box at the bottom represents the average methylation. The overall percentage of methylation for each promoter is noted to the right of each panel. Abbreviations: BM-MSCs, bone marrow-derived mesenchymal stromal cells; HUCPVCs, human umbilical cord perivascular cells; TSS, transcriptional start site.

not influenced by the origin of MSCs but differ from those of hematopoietic progenitor cells versus ESCs [24]. It is well-known that human ESCs are predominantly demethylated on *OCT4* and *NANOG* promoters (5%), while fibroblasts show prominent methylation at these loci (~80%) [9]. It is noteworthy that the degree of methylation at these loci in MSCs is greater than for ESCs but less than for fibroblasts, inferring that the multipotentiality of MSCs as a group may be epigenetically restricted. This mechanism may explain the lower differentiation capacity, and possibly, the very low-risk of tumor formation (if any) of MSCs compared with ESCs.

Conclusion

In conclusion, we showed that HUCPVCs present higher levels of $CD146^+$ stromal progenitors and exhibit enhanced expression of telomerase and pluripotency factors compared with BM-MSCs. Our results also suggest that the methylation of *OCT4* and *NANOG* promoters is a potentially important mechanism in restricting the differentiation capacity of MSCs compared with ESCs.

Acknowledgments

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Disclosure of Potential Conflicts of Interest

The HUCPVC technology, developed at the University of Toronto, was licensed by the University to Tissue Regeneration

Therapeutics Inc. (TRT). TRT supplied the HUCPVCs used in this study. J.E.D. serves on the board of TRT, is a stockholder and is president of the company. A.K. serves as a consultant to TRT and is a minor stockholder.

REFERENCES

1 Sarugaser R, Lickorish D, Baksh D et al. Human umbilical cord perivascular (HUCPV) cells: A source of mesenchymal progenitors. Stem Cells 2005;23:220–229.
2 Khan M, Mohsin S, Khan SN et al. Repair of senescent myocardium by mesenchymal stem cells is dependent on the age of donor mice. J Cell Mol Med 2011;15:1515–1527.
3 Hermann A, List C, Habisch HJ et al. Age-dependent neuroectodermal differentiation capacity of human mesenchymal stromal cells: Limitations for autologous cell replacement strategies. Cytotherapy 2010;12: 17–30.
4 Sarugaser R, Hanoun L, Keating A et al. Human mesenchymal stem cells self-renew and differentiate according to a deterministic hierarchy. PLoS One 2009;4:e6498.
5 Silva J, Smith A. Capturing pluripotency. Cell 2008;132:532–536.
6 Greco SJ, Liu K, Rameshwar P. Functional similarities among genes regulated by OCT4 in human mesenchymal and embryonic stem cells. Stem Cells 2007;25:3143–3154.
7 Herbert BS, Hochreiter AE, Wright WE et al. Nonradioactive detection of telomerase activity using the telomeric repeat amplification protocol. Nat Protoc 2006;1:1583–1590.
8 O'Callaghan N, Dhillon V, Thomas P et al. A quantitative real-time PCR method for absolute telomere length. Biotechniques 2008;44: 807–809.
9 Park IH, Zhao R, West JA et al. Reprogramming of human somatic cells to pluripotency with defined factors. Nature 2008;451:141–146.
10 Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 2001;25:402–408.
11 Sacchetti B, Funari A, Michienzi S et al. Self-renewing osteoprogenitors in bone marrow sinusoids can organize a hematopoietic microenvironment. Cell 2007;131:324–336.
12 Hiyama E, Hiyama K. Telomere and telomerase in stem cells. Br J Cancer 2007;96:1020–1024.
13 Liu L, DiGirolamo CM, Navarro PA et al. Telomerase deficiency impairs differentiation of mesenchymal stem cells. Exp Cell Res 2004;294:1–8.
14 Simonsen JL, Rosada C, Serakinci N et al. Telomerase expression extends the proliferative life-span and maintains the osteogenic potential of human bone marrow stromal cells. Nat Biotechnol 2002;20: 592–596.
15 Sahin E, Depinho RA. Linking functional decline of telomeres, mitochondria and stem cells during ageing. Nature 2010;464:520–528.
16 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.
17 Ramkisoensing AA, Pijnappels DA, Askar SF et al. Human embryonic and fetal mesenchymal stem cells differentiate toward three different cardiac lineages in contrast to their adult counterparts. PLoS One 2011;6:e24164.
18 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.
19 Emrani H, Davies JE. Umbilical cord perivascular cells: A mesenchymal cell source for treatment of tendon injuries. Open Tissue Eng Regen Med J 2011;4:112–119.
20 Dayan V, Yannarelli G, Billia F et al. Mesenchymal stromal cells mediate a switch to alternatively activated monocytes/macrophages after acute myocardial infarction. Basic Res Cardiol 2011;106: 1299–1310.
21 Griffin MD, Ritter T, Mahon BP. Immunological aspects of allogeneic mesenchymal stem cell therapies. Hum Gene Ther 2010;21: 1641–1655.
22 Saxe JP, Tomilin A, Schöler HR et al. Post-translational regulation of Oct4 transcriptional activity. PLoS One 2009;4:e4467.
23 Altun G, Loring JF, Laurent LC. DNA methylation in embryonic stem cells. J Cell Biochem 2010;109:1–6.
24 Sørensen AL, Timoskainen S, West FD et al. Lineage-specific promoter DNA methylation patterns segregate adult progenitor cell types. Stem Cells Dev 2010;19:1257–1266.

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