Induced Pluripotent Stem Cell-Derived Mesenchymal Stromal Cells Are Functionally and Genetically Different From Bone Marrow-Derived Mesenchymal Stromal Cells

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Key Words. Mesenchymal stromal cells • Induced pluripotent stem cells • iPSC-derived mesenchymal stromal cells • Vascular progenitor cells

ABSTRACT

There has been considerable interest in the generation of functional mesenchymal stromal cell (MSC) preparations from induced pluripotent stem cells (iPSCs) and this is now regarded as a potential source of unlimited, standardized, high-quality cells for therapeutic applications in regenerative medicine. Although iMSCs meet minimal criteria for defining MSCs in terms of marker expression, there are substantial differences in terms of trilineage potential, specifically a marked reduction in chondrogenic and adipogenic propensity in iMSCs compared with bone marrow-derived (BM) MSCs. To reveal the cellular basis underlying these differences, we conducted phenotypic, functional, and genetic comparisons between iMSCs and BM-MSCs. We found that iMSCs express very high levels of both KDR and MSX2 compared with BM-MSCs. In addition, BM-MSCs had significantly higher levels of PDGFRα. These distinct gene expression profiles were maintained during culture expansion, suggesting that prepared iMSCs are more closely related to vascular progenitor cells (VPCs). Although VPCs can differentiate along the chondrogenic, osteogenic, and adipogenic pathways, they require different inductive conditions compared with BM-MSCs. These observations suggest to us that iMSCs, based on current widely used preparation protocols, do not represent a true alternative to primary MSCs isolated from BM. Furthermore, this study highlights the fact that high levels of expression of typical MSC markers such as CD73, CD90, and CD105 are insufficient to distinguish MSCs from other mesodermal progenitors in differentiated induced pluripotent stem cell cultures.

SIGNIFICANCE STATEMENT

The generation of mesenchymal stromal cells (MSCs) from iMSCs has been proposed as an alternative strategy for obtaining unlimited sources of these cells of defined quality and capable of meeting increasing demands for research and therapeutic applications. However, there are distinctions between primary MSCs and iMSCs in terms of phenotype and differentiation. This study shows that iMSCs share phenotypic traits with bone marrow-derived (BM)-MSCs in terms of standard marker expression but shows marked differences in gene expression, especially in genes associated with vascular progenitor cells. These differences may explain the altered differentiation propensity of human iMSCs compared with primary MSCs.

INTRODUCTION

The discovery of induced pluripotent stem cells (iPSCs) has been a key breakthrough in stem cell biology and regenerative medicine. The properties of pluripotent differentiation and long-term self-renewal in vitro [1] exhibited by these cells are of great significance and potential value in developing new therapeutic strategies without encountering ethical obstacles associated with embryonic stem cells (ESCs). iPSCs have the same genetic background as the somatic cells from which they were derived, giving rise to the prospect of generating constantly renewable immunoprivileged cell populations for autologous cell therapy [2]. Another opportunity of value is provided by the prospect of using iPSCs to uncover the origin and pathogenesis of human diseases [3, 4].

One aspect of iPSC technology that has drawn considerable interest has been the generation of functional mesenchymal stromal cell (MSC) preparations. This has arisen because of the strong interest in the research and therapeutic use of these cells. MSCs are multipotent cells...
which have been extensively tested as therapeutic agents for the treatment of degenerative diseases, autoimmune disorders and wound repair [5, 6]. In addition, the cells can be used to model human diseases, for example, musculoskeletal disorders [7] and nonhematological mesenchymal cancers [8]. MSCs are easily accessible in postnatal tissues, including bone marrow (BM), adipose tissue, and umbilical cord blood [9]. However, problems with the inconsistency of supply, variable quality, and phenotypic changes associated with long-term expansion have represented impediments to effective translation [10]. To overcome these barriers, iPSCs, with the advantages mentioned above, have the potential to become a more standardized alternative to primary tissues for MSC production.

Human pluripotent stem cell-derived MSCs (PSC-MSCs) were first differentiated from ESCs, referred to ESC-MSCs, using a strategy that involved differentiation of cells within the embryoid body (EB) [11]. In recent years, protocols have also been developed for the generation of MSCs from human iPSCs. Commonly used protocols included: (a) transferring iPSCs to a suspension culture to form EBs followed by culturing on gelatin-coated plates [12]; (b) directly seeding dissociated iPSC colonies in precoated culture surfaces such as gelatin, collagen type 1, or synthetic polymers [13–15]; or (c) simply replacing iPSC culture medium with MSC medium [16]. Furthermore, inhibitors of kappaB kinase epsilon (IKKε), the TGF-β/Activin type I receptor inhibitor (SB431542), Activin A, and BMP4 have been used to enhance early mesodermal induction in culture [17, 18]. iPSC derivatives were termed iMSCs, because of their MSC-like immunophenotype, plastic adherent ability, and apparent multipotent differentiation potential in chondro-, osteo-, and adipogenesis, therefore meeting the minimal criteria defined by the International Society for Cellular Therapy (ISCT standard) [19].

However, questions arise relating to the phenotypic fidelity of iMSCs because, irrespective of the derivation methods used, there are differences between these cells and primary MSCs. For instance, iMSCs generally have less capacity for adipogenic and chondrogenic differentiation. Adipocytes and chondrocytes differentiated from iMSCs often express significantly lower levels of lineage marker genes such as PPARγ and ADIPOQ (adipogenesis) and ACAN and COL2A1 (chondrogenesis) than those derived from primary MSCs, [20–25]. Conversely, iMSCs are markedly efficient in osteogenesis based on the evaluation of matrix production and osteogenic marker expression [26–29]. The altered differentiation propensity may hinder the application of iMSCs in current research and therapeutic strategies such as those involving primary MSCs for disease modeling and tissue regeneration.

Previous hierarchical analysis of gene expression profiles (GEPs) suggested that both iMSCs and primary MSCs have the characteristics of mesodermal lineage but are clearly not identical. Gene clustering analysis showed that, irrespective of the differentiation methods used, iMSCs formed a cluster which was close to but separated from the primary MSC group [20]. Moreover, Frobel et al. demonstrated the dissimilarity in DNA methylation patterns between the two cell types [21]. However, the significance of the distinct GEPs between iMSCs and primary MSCs, and the possible relationship to differences in multipotency remain poorly understood.

To answer these questions, we compared the differentiation ability, immunophenotype, and GEPs between multiple iMSCs and BM-MSC lines by looking at key genes representing different mesodermal stem cell populations. The phenotype, multipotency, and GEP of iMSCs in serial passages were also assessed to evaluate the impact of culture expansion. Our results showed that iMSCs demonstrated equivalent osteogenicity but less adipogenicity and chondrogenicity when compared with BM-MSCs. The GEPs of the two cell groups were significantly different and such distinction was maintained consistently during culture expansion, suggesting that both cell types represented different mesodermal progenitors and that iMSCs were, in fact, more similar to vascular progenitor cells (VPCs). Previous findings showed that even though the cell plasticity of VPCs endows them with capacities to undergo chondro-, adipo-, and osteogenesis, specific conditions are required to fully induce the lineage transformation of the cells, especially for chondrogenesis and adipogenesis [30–34]. This may explain why iMSCs with VPC background showed an MSC-like multipotent potential but displayed a functional inadequacy in a MSC preferred differentiation environment. In addition, a partially overlapped surface profile between VPCs and MSCs [35, 36] can make them indistinguishable by examining the markers selected in the ISCT standard. Taken together, our results suggested that it is insufficient to apply the ISCT standard alone to precisely define MSC populations derived from iPSCs. To reduce the risk of lineage misidentification, it is necessary to further validate the cell identity with various mesodermal progenitor markers.

**Materials and Methods**

**Ethical Approvals**

To isolate primary MSCs, BM was harvested from the iliac crest of three healthy donors with approval from both the NUI Galway Research Ethical and Galway University Hospitals Clinical Research Ethics Committees. Skin fibroblasts for generation of iPSC-3 were obtained from a healthy donor with ethical approval by the University Hospital Galway research ethics committee.

**Cell Lines**

BM-MSC lines from three healthy donors were isolated and cultured as previously described [37]. iPSC-1 and iPSC-2 were kindly provided by Dr. Gareth Sullivan (University of Oslo) and Professor James Dutton (University of Minnesota), respectively. The conditions used for cell expansion and iPSC characterization protocols are included in Supporting Information.

**Generation of iMSCs**

The EB outgrowth method was applied as described previously with minor modifications. EBs were maintained in suspension culture for 8 days, followed by adherent culture on gelatin-coated plates for outgrowth of differentiated cells. During this stage, EB formation medium was used which contained 80% knockout Dulbecco’s modified Eagle’s medium (KO-DMEM, Invitrogen, Glasgow, UK), 20% fetal bovine serum (FBS; Sigma), 1 mM L-glutamine (Invitrogen, Glasgow, UK), 14 μM β-mercaptoethanol (Invitrogen, Glasgow, UK), and 1% nonessential amino acids (NEAA, Invitrogen, Glasgow, UK) [11]. When outgrown cells formed confluent areas, they were passaged to new gelatin-coated flasks and remaining EB clumps were removed using a 40 μm cell strainer. During this maintenance phase, cells were fed with medium comprising KO-DMEM, 10% FBS, 1 mM L-glutamine, and 1% NEAA [38]. Finally, when the majority of the cells displayed an MSC-like morphology, they were transferred to...
noncoated culture flasks with MSC culture medium (passage 0 [P0]). A continued homogeneous transition was achieved in each iPSC culture by eliminating nonadherent cells.

**Proliferative Potential and Immunophenotypic Assessment**

Cell growth rate was determined as cumulative population doubling (CPD) [39].

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CPD = \log\left(\frac{\text{No. harvested}}{\text{No. seeding}}\right)/\log(2)
\]

For immunophenotyping, flow cytometry analysis was performed using the human MSC analysis kit (BD Bioscience, San Diego, CA) according to the manufacturer’s protocol. Individual antibodies against CD11b, CD19, CD45, CD34 (BD Bioscience, San Diego, CA), and human leukocyte antigen (HLA)-DR (Thermo Fisher Scientific, MD) were also included in the analysis. Samples were analyzed on the BD FACS Canto II (BD Biosciences) and data analyzed using FlowJo 6.0.

**Trilineage Differentiation**

MSCs were subjected to chondro-, adipo-, and osteogenesis as described previously with minor modifications [37]. Differentiated cells were analyzed by histological and immunocytochemical staining. The differentiation efficiency was also assessed by quantitation of related biomarkers. Detailed experimental procedures can be found in Supporting Information.

**GEP Analysis**

Real-time quantitative polymerase chain reaction (qPCR) and semiquantitative PCR were performed to evaluate the GEP of the MSCs. Protocols for sample preparation and PCR analysis can be found in Supporting Information. Information relating to primers is given in Supporting Information Table S1.

**Statistical Analysis**

All values are presented as a mean ± SEM. Two-tailed Student’s t test was performed to detect the statistical differences, in gene expression, immunophenotype, and differentiation efficiency, between BM-MSCs and iPMSCs at the same passage. To detect differences in expression variation of the selected gene of the same cell type during culture expansion, One-way analysis of variance followed by Bonferroni (compare all pairs of columns) correction were performed. \( p < .05 \) was considered statistically significant.

**Results**

**Generation and Characterization of iPSC Lines**

Three iPSC lines were included in this study, iPSC-1, 2, and 3. All lines were derived from healthy donors using the standard retroviral reprogramming method [38]. The pluripotent state of iPSC-1 was validated previously [7] and characterization of iPSC-2 and 3 included assessment of typical ESC-like morphology (Supporting Information Fig. S1A), confirmation of a normal karyotype (Supporting Information Fig. S1B) and immunostaining to identify positive expression of key pluripotent markers OCT4, SOX2, NANOG, and TRA1-60 (Supporting Information Fig. S1C). Furthermore, teratoma assay and EB formation were applied to examine the functional pluripotency of iPSC-2 and 3, respectively. Histological analysis of teratoma sections derived from iPSC-2 showed that the cells were able to generate different tissue types from three germ layers (Supporting Information Fig. S1D), whereas immunostaining of day 35 EBs confirmed pluripotency of iPSC-3 with positive staining for α-SMA (mesoderm), α-Fetoprotein (endoderm), and β-3-tubulin (ectoderm; Fig. 1A–1E).

**Phenotypic Comparison of iMSCs and BM-MSCs**

Since the EB outgrowth method is the most widely studied for the differentiation of iPSCs to MSCs [17], it was used in this study. In addition, three primary MSC lines were obtained from BM aspirates from the iliac crest of healthy donors. P5 was chosen as the earliest time point for analyzing iPMSCs because earlier passage cells showed heterogeneous morphology and limited plastic adherence. The impact of serial passages on phenotype, differentiation and GEP was studied between P5 and P8 in both iPMSCs and BM-MSCs.

Preliminary analysis was carried out to compare the morphology, proliferative activity, and surface antigen expression between the two groups. Cell morphology was similar across all preparations (Fig. 1A) and there was no significant difference between iPMSCs and BM-MSCs in terms of their proliferative capacity (Supporting Information Fig. S2), both showing similar mean CPD (Fig. 1B). Immunophenotypic analysis confirmed that both cell groups expressed standard MSCs surface markers (CD73/90/105; Fig. 1C). Some preparations showed some positive expression of hematopoietic markers (Fig. 1C). When this was observed, we undertook a detailed analysis and found that it was specifically associated with HLA-DR expression only and the cells were negative for other hematopoietic markers (CD11b/34/19/45; Fig. 1E; Supporting Information Fig. S3B, S3C). We concluded that the preparations did not contain hematopoietic progenitors and that the induction of HLA-DR expression was a consequence of expansion in the FGF2-containing medium, as described previously [40]. We also noticed that iPMSCs contained significantly less CD90⁺ cells than BM-MSCs (\( p = .0038 \); Fig. 1D) and a bimodal pattern of CD90 expression was observed in iPMSC preparations only (Fig. 1C). This appeared to be a consequence of changed CD90 expression in some cultures with passaging (Supporting Information Fig. S3A).

**Functional Comparison of iMSCs and BM-MSCs**

The capacity for trilineage differentiation in both cell groups was assessed using standard differentiation conditions. Consistent with previous findings [20, 21], we observed that iPMSCs demonstrated limited adipogenic differentiation. In adipocytes derived from BM-MSCs, Oil Red O-stained lipid droplets (LDs) were much larger compared with those from iPMSCs (Fig. 2A). Consistently, the level of triacylglycerol (TAG) was significantly lower in iPMSCs (20-fold, \( p = .0056 \), Fig. 2B). Interestingly, the higher DNA content seen in differentiated iMSC cultures suggests potential differences in cell cycle regulation and proliferative activity between the two groups (Fig. 2C). The significantly lower ratio of TAG per DNA suggested a disrupted TAG synthesis in iPMSCs compared with BM-MSCs (40-fold, \( p = .003 \), Fig. 2D). This difference remained in both early passage (P5; \( p = .006 \)) and late (P8; \( p = .004 \)) cultures (Fig. 2E), further indicating an incomplete adipogenic capacity of iPMSCs.

Detailed analysis of chondrogenic potential revealed striking differences between iMSCs and BM-MSCs. Toluidine blue staining showed an abundant glycosaminoglycan (GAG)-rich extracellular
Figure 1. Preliminary characterization of induced mesenchymal stromal cells (iMSCs) and bone marrow (BM) derived-MSCs. (A): Cellular morphology. Scale bar = 50 μm. (B): Proliferation capacity of each cell group compared based on the cumulative population doublings (CPD) (mean ± SEM). See Supporting Information Figure S2 for the growth rate of each line. (C–E): Surface profile analysis of the cells (P5) using flow cytometry. Red histograms represent isotype controls with the blue overlays representing each antigen; percentages of positive cells are shown within histograms. (C): Positive markers included CD90/73/105. The negative (Neg.) combine is a mixed antibody pool assessing CD45, CD34, CD19, CD11b, or HLA-DR positive cells. (D): Summary of the immunophenotype of BM-MSC and iMSC lines as detected by flow cytometry. Data represent mean ± SEM of n = 3; * p < .05. (E): Individual antibody staining of hematopoietic populations to identify the positive signal shown in the Neg. combine group. See also Supporting Information Figure S3.
matrix (ECM) in 35 days chondrogenic pellets of BM-MSC and very limited GAG staining in iMSC pellets (Fig. 3A). Measurement of the accumulated GAG to DNA ratio (Fig. 3B) showed that BM-MSCs produced 6.7-fold higher GAG at P5 ($p = .008$) and 14-fold higher at P8 ($p = .01$).

Immunohistological analysis of specific ECM components underscored the differences in chondrogenic response between BM-MSCs and iMSCs (Fig. 3C). This showed that collagen type II (COL2), collagen type VI (COL6), cartilage oligomeric matrix protein (COMP) and aggrecan were synthesized at a high level and distributed throughout the ECM in BM-MSC derived pellets. However, aggrecan, COL2, and COMP, hallmarks of a cartilaginous phenotype, were absent or minimally expressed in iMSC pellets. COL6 was the only ECM component that appeared to be expressed at a high level in iMSC pellets and this was not uniformly distributed. Taken together, these results suggested a very limited chondrogenic proclivity of iMSCs.

In contrast, both iMSCs and BM-MSCs demonstrated strong osteogenic capacity as measured by calcium deposition, osteocalcin synthesis, and assembly of a collagen type I (COL1)-rich ECM. Osteocalcin was expressed abundantly in cell aggregates maintained in osteogenic medium for 35 days (Fig. 4A). The osteogenic cultures from both cell groups also showed similar abundant accumulation of calcium measured relative to total DNA content (Fig. 4B). In addition, analysis of COL1, the major collagen in bone [41], showed a richer accumulation in iMSC-derived cultures compared with BM-MSC-derived cultures (Fig. 4C).

**iMSCs Were Derived Through Mesodermal Commitment**

To interrogate the possibility that lack of trilineage differentiation capacity could be associated with the presence of heterogeneous mixtures of cells of nonmesodermal lineage, the expression of genes representing ectoderm and endoderm was assessed (Fig. 5).

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**Figure 2.** Adipogenic capacity of induced mesenchymal stromal cells (iMSCs) and bone marrow (BM) derived-MSCs. (A): Morphologies of formed lipid droplets shown by staining with Oil Red O solution. Representative images of each cell line (P5) are shown. Insets are magnified images of the areas indicated by black arrows. Scale bar = 22.7 μm. (B–D): The amount of TAG and DNA and the ratio of TAG per DNA between the BM-MSCs and iMSCs groups are shown. Data in each bar chart represents mean ± SEM of $n = 3$; **, $p < .01$. (E): Quantification of lipid elaborated by extraction of Oil Red O stain and measuring absorbance at O.D. 490 nm, $n = 3$ wells for each group. Results are mean ± SEM; **, $p < .01$. ©2019 The Authors. *STEM CELLS* published by Wiley Periodicals, Inc. on behalf of AlphaMed Press 2019
PCR analysis showed no expression of PAX6 and SOX1 indicating that cells of neuroectodermal lineages were not present in iMSCs and BM-MSCs [42]. Similarly, the absence of FOXA2 and CXCR4 indicated that no definitive endoderm was present. We did note, however, that the early regulators of endodermal differentiation, SOX17 and SOX7 [43], were detected in iMSCs in P5 but absent in later passage cells (Fig. 5). These results suggested that differences in phenotype or differentiation potential between MSCs derived from iPSC and from BM could not be attributed to the presence of nonmesodermal lineage cells.

We next considered whether iMSCs and BM-MSCs differed in terms of early or late mesodermal commitment. Previous studies have suggested that iMSCs are less mature than BM-MSCs and that this may influence differentiation capacity [44]. To verify this, we selected a set of markers that would reveal the degree of mesodermal commitment for each group. We compared the expression levels of MIXL1, MESP2, and CDH1, all of which are involved in the regulation of early mesodermal commitment and differentiation. MIXL1 is mainly expressed in nascent mesoderm followed by downregulation at the point of commitment [45, 46]. MESP2 is activated on initiation of segmentation in paraxial mesoderm (PM) [47]. CDH1 is a marker of endomesoderm and its expression pattern is reduced during mesogenesis but maintained for endogenesis [48, 49].

There was no significant difference in expression of MIXL1 in iMSCs and BM-MSCs over several passages in culture (from P5 to P8, Fig. 6A). BM-MSCs showed higher expression of MESP2 compared with iMSCs across all passages. These differences were

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**Figure 3.** Comparison of chondrogenic pellets derived from induced mesenchymal stromal cells (iMSCs) and bone marrow (BM) derived-MSCs. (A): Representative images of toluidine blue stained sections demonstrate the presence of GAG within the chondrogenic matrix of the pellets derived from MSCs at P5. Scale bar = 250 μm. (B): GAG quantification relative to DNA content of tested pellets. Data represents mean ± SEM of n = 3; **, p < .01. (C): Representative images of immunofluorescent staining for cartilaginous matrix components (aggrecan [red], COMP [green], COL2 [red], and COL6 [green]) on the pellets differentiated from P5 MSCs. Cell nuclei were stained with DAPI (blue). Scale bar = 50 μm. Insets displayed in (A) and (C) are magnified images of the areas indicated by the arrowheads.

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not significant except for P5 (p = .0068; Fig. 6B). This may suggest a PM origin of BM-MSCs. In contrast to BM-MSCs, iMSCs expressed significantly lower levels of CDH1 at P7 (20-fold, p = .0221) and P8 (10-fold, p = .0028; Fig. 6C). The marked change may be associated with maturation to a mesoderm state.

Conversely, the high expression of both CDH1 and SOX7 in BM-MSCs (Fig. 5) may infer the existence of an endodermal precursor which is in line with the potential of these cells to transdifferentiate into endodermal lineages [50]. Overall, a similarly low level of MIXL1 detected on both cell types implies their
postmesodermal initiation state. Contrasting expression patterns of MESP2 indicate that iMSCs and BM-MSCs may arise from different mesodermal subfractions.

iMSCs and BM-MSCs Represent Distinct Mesodermal Progenitors

Next, we looked at the expression of genes that are widely used to distinguish subpopulations of mesodermal progenitors. We noted a dramatically higher level of PDGFRα expression by BM-MSCs compared with iMSCs at all passages: P5 (24-fold, \( p = .036 \)), P6 (28-fold, \( p = .0702 \)), P7 (40-fold, \( p = .0042 \)), and P8 (55-fold, \( p = .0028 \); Fig. 6D). A gradual diminution in the level of PDGFRα (39% over time) was also seen in iMSCs on passaging. Conversely, KDR expression was greater in iMSCs than in BM-MSCs from 4-fold (P5, \( p = .07 \)) to 223-fold (P8, \( p = .1 \); Fig. 6E).

PDGFRα and KDR are restricted to PM and lateral plate mesoderm (LPM), respectively, during mesogenesis [51, 52]. Recently, PDGFRα has emerged as a promising marker for isolation of primary MSCs from BM and heart [51]. Overall, the very low level of PDGFRα in iMSCs suggests that MSC-like cells are rare in iMSC cultures whereas the high expression of KDR suggests that iMSCs may originate in LPM.

In both human and mouse models, ESC-derived KDR+ stem cells have been recognized as hemangioblasts, giving rise to hematopoietic lineages and VPCs [53]. Based on flow cytometry analysis, the hematopoietic markers were expressed at extremely low levels in iMSCs (Fig. 1; Supporting Information Fig. S3). Therefore, we speculate that KDRhigh iMSCs may be VPC-like cells. The significantly stronger expression of MSX2 in iMSCs further supports this conjecture (Fig. 6F). Goupille et al. used nlacZ as a reporter for Msx2 expression in a transgenic mouse model and showed that this gene was expressed prominently in the vascular smooth muscle layer of peripheral arteries as well as in embryonic and adult aorta endothelium [54]. Our results showed that the overall expression levels of MSX2 in iMSC group were 37.5- (P8) to 100-fold (P5) higher than the levels in BM-MSCs. Statistical significance was achieved by P6 (65.7-fold, \( p = .046 \); Fig. 6F). In addition, the consistently high level of MSX2 in iMSCs may suggest a stable VPC phenotype [55]. Furthermore, the unique GEP of iMSCs suggests a potential differentiation propensity to endothelial precursors and vascular smooth muscle progenitors [56].

Taken together, the results suggest that iMSCs are mature mesodermal progenitors with a VPC-like background and that iMSCs and BM-MSCs represent different mesodermal progenitor populations (Fig. 7). The proposal that iMSCs have a VPC-like background provides a rationale for their functional divergence from BM-MSCs.

**DISCUSSION**

In this study, the iPSC derivatives were termed as iMSCs as the cells possessed the characteristics of MSCs as described by the ISCT standard (Figs. 1–4). However, the GEP analysis performed argued that these two cell groups actually represented different mesodermal subfractions, where iMSCs were VPC-like cells (PDGFRαlow KDRhigh MSX2high) and BM-MSCs were a product of the mesengenic process (PDGFRαhigh KDRlow MSX2low; Fig. 6). The current understanding of VPCs provides the rationale for explaining inconsistencies between iMSCs and BM-MSCs, that is, the compliance of iMSCs to the ISCT-based cell surface phenotype and noncompliance with respect to differentiation capacity.

Firstly, flow cytometry analysis showed that iMSCs carried a BM-MSC-like surface profile (Fig. 1C). Previous findings proved that VPCs positively expressed not only CD73/90/105 but also CD44/166 and STRO-1 [57–62]. Due to the partially overlapping immunophenotype found, using these traditional MSC-associated antigens contributes to an inability to discriminate iMSC with a VPC-like background from the MSC population. Interestingly, the
coexistence of CD90\textsuperscript{high} and CD90\textsuperscript{low} subsets was captured exclusively in iMSC lines (Fig. 1C). Of note, this immunophenotype was also observed in PSC-MSC lines from other studies [13, 15, 29, 63–65]. We found that continued expansion could induce a dynamic expression pattern of CD90 in the cells (Supporting Information Fig. S3A). Although Moraes et al. showed that depletion of CD90 in BM-MSCs stimulated their osteogenic ability [66], this association was not found in iMSCs generated in our study (Fig. 4B). An alternate role of CD90 in adult cardiac vascular stem cells (CVSCs) was investigated by Gago-Lopez et al., who reported that CD90\textsuperscript{−} CVSCs expressed higher levels of genes involved in stemness when compared with CD90\textsuperscript{+} CVSCs [67]. Whether a similar influence of CD90 variation can be found in iMSCs will need to be elucidated.

Secondly, both cell groups showed marked potential in trilineage differentiation. However, in contrast to BM-MSCs, iMSCs were found to have significant osteogenic capacity; chondrogenicity and adipogenicity were minimal (Figs. 2–4). This differentiation phenotype corresponds with the known fate plasticity of VPCs. It has been shown that VPCs display osteogenic capacity, which can be differentiated into osteoblasts in a primary MSC preferred condition, using β-glycerol phosphate, ascorbic acid, and dexamethasone [30, 68, 69]. However, for chondrogenesis, the cells require an additional treatment of BMP2, BMP4, or TGF-β2 to complete a mesenchymal transition by interacting with members of the type II TGF-β receptor [30, 32, 70, 71]. In our study, TGF-β3, as one of the commonest chondrogenic inducer for primary MSCs, was used for chondrogenesis of iMSCs. Although previous ligand-receptor binding analysis demonstrated binding ability between TGF-β3 and the type I receptor, this ligand has stronger binding preference and affinity to the type II receptor [72]. Uniquely, CD105, a type III TGF-β receptor, also binds TGF-β3 with high affinity but fails to bind TGF-β2, BMP2, and BMP4 [73, 74]. The distinct receptor preference of TGF-β3 may explain the insufficient chondrogenesis of iMSCs. With reference to adipogenesis, Zhao et al. showed that tissue derived KDR\textsuperscript{+} CD31\textsuperscript{+}CD34\textsuperscript{−} cells, with characteristics of VPCs, were capable of adipogenesis [31, 33]. Zimmerlin et al. compared the adipogenic capacity among vascular lineages and showed that the differentiation efficiency was low in CD31\textsuperscript{+}endothelial lineages but high in CD146\textsuperscript{−}smooth muscle cells or pericytes [34]. These findings confirmed that VPCs have adipogenic potential but also implied that a pre-VSMPs derivation of iMSCs may help to enhance their adipogenic efficiency. Taken together, the partially overlapped multipotency and disparate requirements for differentiation conditions between VPCs and MSCs provide the rationale to explain why iMSCs with a VPC-like background showed the potential for trilineage differentiation but were dissimilar in differentiation efficiency when compared with BM-MSCs.

GEP examination is a very important strategy for verifying cell type after differentiation of stem cells. In this study, redefining the lineage type of iMSCs based on their GEPs helped to explain the functional variation between the cells and BM-MSCs. However, the number of publications including both GEP and functional comparison between PSC-MSCs and primary MSCs is limited. Data reported previously indicated that PSC-MSCs with functional inequivalence to primary MSCs commonly expressed high levels of genes associated with disparate mesodermal progenitors but exceedingly low levels of key MSC genes. Barbet et al. showed that in comparison to BM-MSCs, ESC-MSCs derived by a spontaneous differentiation method carried significantly higher levels of HAND1 (LPM marker) [75], NKK2-5, and FLT4 (cardiovascular progenitor genes) [44, 76]. In Billing et al.’s study, mesengenic derivation of ESCs directly in MSC maintenance medium led to the high expression of NKK2-5 and FGF12 (a promoter of vascular smooth muscle commitment) [77], which were exclusively detected in the ESC-MSCs [78]. Diederichs and Tuan generated MSCs using these methods on iPSCs and demonstrated that KDR was significantly higher in the resultant cells than in BM-MSCs. In addition, their data showed that the iPSC derivatives also expressed significantly higher MCAM, the elevation of which is associated with vascular smooth muscle transdifferentiation of primary MSCs [20, 79]. Inefficient mesengenic induction was also seen even when the process was derived in a controlled manner. Mesengenesis of iPSCs by inhibiting TGF-β/Activin/Nodal signaling with SB-431542 resulted in a greater expression of VEGFR1 (FLT1), a marker of vascular endothelial cells in iMSCs than in BM-MSCs [28, 80]. Mesengenic induction using FGF2 and PDGF followed by cell sorting for a CD105\textsuperscript{+}CD24\textsuperscript{−} population also gave rise to a heterogeneous culture with greater expression of vascular endothelial markers (CL-P1 [81] and FAM43A [82]) and neuroprogenitor markers (EFNB3 [83], SLITRKS [84], and Ckap2l [85]) in...
ESC-MSCs than in BM-MSCs [86]. Moreover, irrespective of the derivation methods used, extremely low expression of MSC-associated genes was often detected in these PSC-MSCs, including IL6 [20, 21, 28], BST1 [21, 44, 86], SFRP4 [21, 44], and VCAM1 [21, 44, 86]. The mismatches in GEPs between PSC-MSCs and primary MSCs observed in other studies strongly support our speculation on the lack of efficiency of the commonly used differentiation methods in inducing the mesengenic specification of PSCs. Subsequently, this uncontrolled lineage commitment can lead to a generation of nonspecific mesodermal progenitor populations.

CONCLUSION

Our data clearly demonstrated that the ISCT standard is insufficient to precisely distinguish MSCs from other mesodermal progenitors. Distinct GEPs can be overlooked as a result of partially overlapping cellular characteristics between MSCs and other mesodermal progenitors. Inaccurate lineage identification as a result of incomplete characterization can compromise the ability to compare and contrast study outcomes and may be responsible for functional discrepancies of iMSC seen in other related studies. Additional examination of markers representing different mesodermal progenitors should be performed as a standard process for tracking differentiation and verifying the iMSC identity before used in future investigations.

On the other hand, to further validate the VPC background of iMSCs shown here, future analysis can be conducted to compare differentiation propensity, immunomodulatory activity and GEPs among iMSCs, BM-MSCs, and tissue-derived VPCs. It will also be of interest to investigate whether the cell origin of iPSCs can influence their mesengenic efficiency.

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