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Feeder-free generation and transcriptome characterization of functional mesenchymal stromal cells from human pluripotent stem cells

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ABSTRACT

Induced mesenchymal stromal cells (iMSCs) derived from human pluripotent stem cells (PSCs) are attractive cells for regenerative medicine. However, the transcriptome of iMSCs and signature genes that can distinguish MSCs from fibroblasts and other cell types are rarely explored. In this study, we reported an optimized feeder-free method for the generation of iMSCs from human pluripotent stem cells. These iMSCs display a typical MSC morphology, express classic MSC markers (CD29, CD44, CD73, CD90, CD105, CD166), are negative for lymphocyte markers (CD11b, CD14, CD31, CD34, CD45, HLA-DR), and are potent for osteogenic and chondrogenic differentiation. Using genome-wide transcriptome profiling, we created an easily accessible transcriptome reference for the process of differentiating PSCs into iMSCs. The iMSC transcriptome reference revealed clear patterns in the silencing of pluripotency genes, activation of lineage commitment genes, and activation of mesenchymal genes during iMSC generation. All previously known positive and negative markers for MSCs were confirmed by our iMSC transcriptomic reference, and most importantly, gene classification and time course analysis identified 52 genes including FN1, TGFB1, TAGLN and SERPINE1, which showed significantly higher expression in MSCs (over 3 folds) than fibroblasts and other cell types. Taken together, these results provide a useful method and important resources for developing and understanding iMSCs in regenerative medicine.

1. Introduction

The mesenchymal stromal cells (MSCs) are promising cell candidates for regenerative medicine and cell therapy (Chen et al., 2008; Tuan et al., 2003), owing to their great self-renewal capability and differentiation potential. Functional MSCs can be isolated from various tissues such as bone marrow (Pittenger et al., 1999), adipose tissue (Zuk et al., 2001), blood (Zvaifler et al., 2000; Schuh et al., 2009; Lee et al., 2004) and muscle (Bosch et al., 2000; Crisan et al., 2008). Protocols for deriving MSCs from exogenous tissues have been well established. However, broader applications of adult MSCs are limited due to the low cell population and inter-tissue difference of MSCs from adult tissues, distinct proliferation capacity and the highly invasive procedure of isolating MSCs (Kern et al., 2006; Yoo et al., 2009). Furthermore, the
proliferation capability and differentiation potential of MSCs derived from bone marrow (BM-MSCs) reduces with the aging of donors (Minguell et al., 2001; Stolzing et al., 2008). Alternatively, an infinite number of induced MSCs (iMSCs) can be derived from pluripotent stem cells such as induced pluripotent stem cells (iPSC) and embryonic stem (ES) cells, providing an alternative cell resource for cell therapeutic application. It has been shown that iMSCs derived from iPSC can be used for investigation of limb ischemia, in which the immunosuppressive properties of iMSC are greater than that of BM-MSCs (Lian et al., 2010; Sun et al., 2015). Meanwhile iMSCs have longer lasting immune-regulatory function and much less potential to promote tumors (Giuliani et al., 2011; Zhao et al., 2015). As for another inexhaustible cell origin, the iMSCs derived from ES cells hold robust proliferation capacity and differentiation potential (Karlsson et al., 2009), immunophenotype similar to BM-MSCs (Barberi et al., 2005). These cells can also support the growth of hematopoietic progenitors (Olivier et al., 2006) and demonstrated a superior neuroprotective potential over fetal MSCs in mouse brain hypoxia–ischemia model (Hawkins et al., 2018).

To date, most iMSCs derivation methods still encounter several technical limitations, such as time-consuming, laborious flow sorting using several surface markers of MSCs (Lian et al., 2010, 2016; Barberi et al., 2005), reduced differentiation capability (Villa-Diaz et al., 2012), additional chemicals/small molecules treatment (Chen et al., 2012) and special culture condition (e.g. 7.5% CO₂) (Zhao et al., 2015), and most importantly the limitation in scalability for large production. In our previous study (Zou et al., 2013), we have established a simple and efficient iMSC derivation method, which involved culturing human iPSCs on a monolayer of mouse feeder cells. However, this method has a potential risk of bringing contaminants from mouse feeder cells, which is a major disadvantage for subsequent applications (Giuliani et al., 2011). Thus, it is necessary to modify the previous method to adapt a feeder-free system. Another advantage of developing the feeder-free system is to enable large scale production of iMSCs and better systematic characterization of iMSCs. To overcome these, we here describe the development and characterization of an optimized method for generating iMSCs from human iPSCs and ES cells using a feeder-free system.

MSCs are known to be a heterogeneous population and the cell heterogeneity is partially attributed to the tissue of origin. Using bulk RNA sequencing Kyung-Ah Cho et al. successfully categorized differentially expressed genes in the whole transcriptome and compared the signaling pathways between MSCs derived from three different tissues. These transcriptome portraits of the MSC populations are very useful to understand the characteristics of MSCs derived from different tissues (Cho et al., 2017). Moreover recent publications have investigated the heterogeneity of MSCs by comparing gene expression profiles at single cell level using single-cell RNA sequencing (scRNA-seq) technology. Yin Huang and the team showed MSCs derived from umbilical cords possessed limited heterogeneity in vitro expansion by profiling the transcriptomes and analyzing the gene network from 361 single MSCs and proved that cell heterogeneity is dominated by cell cycle status (Huang et al., 2019). Xuanyu Liu et al. provided a high-quality dataset by a large-scale scRNA-seq of 24 358 cultured MSCs derived from adipose which is a valuable resource for interrogating the MSCs heterogeneity (Liu et al., 2019). Another publication successfully reported gene-expression heterogeneity and several distinct subpopulations of human primary Wharton’s Jelly-derived MSCs by scRNA-seq (Sun et al., 2019).

Therefore, to understand dynamic changes of gene expression during iMSC generation of human pluripotent stem cells in vitro, we performed a genome-wide transcriptome analysis for the whole process of iMSCs derivation, and generated an easily accessible iMSC gene expression reference in this study. Our results demonstrated that this iMSC transcriptome reference recapitulates the transcriptome dynamics in differentiation and MSC lineage commitment, all the classical positive and negative markers of MSCs, as well as novel signature genes for MSCs.

2. Materials and methods

2.1. Cell culture

Normal human dermal fibroblasts (GM01450) were cultured in fibroblast medium, consisting of DMEM (BE12-604F, LONZA), 10% FBS (F7524, sigma), 2 mM L-glutamine and 1% penicillin/streptomycin. The iPSC and H9ES cells were maintained on mouse feeder cells in iPSC knockout medium: DMEM (10829–018, Gibco) supplemented with 20% KSR (10828–028, Gibco), 1% NEAA (11140–050, Gibco), 0.2% 2-mercaptoethanol (31350–010, Gibco), 1% Glutamax, 1% penicillin/streptomycin and 10 ng/ml BFGF (PHG0026, Invitrogen). In feeder-free culturing system, the iPSC and H9ES cells were cultured onto VTN-N (A14700, Gibco) coated dishes/plates in Essential 8™ Medium (E8, A15170-01, Gibco) and routinely passaged with ReLeSR (05872, Stem Cell Technologies) as cell clumps. iPSC-iMSC, H9ES-iMSC and BM-MSC were all cultured in MSC low glucose growth medium containing DMEM (31885-023, Gibco), 10% highly qualified FBS (26140–079, gibco), 1% penicillin/streptomycin, and 1% Glutamax. H9ES cell was a gift from Associate Professor Mark Denham, Aarhus University.

2.2. Generation of human iPSC

Human dermal fibroblasts (GM01450) from skin biopsy were kindly provided by Associate Professor Thomas Juhl Corydon in Aarhus University. Lentivirus pRRL.PPT.SF.hOct34.hKlf4.hSox2.hmCMV.Tomato (LV-OKSM), which derives a polycistronic cassette of expressing Yamanaka factors (OCT4, KLF4, SOX2, C-MYC) was used for lentivirus-based reprogramming experiment. Fibroblasts at passage 10–15 (P10-P15) were seeded onto 6-well plates at a density of 1.5x10⁵ (cell/well). 24 h post cell seeding, the fibroblasts were transduced with concentrated LV-OKSM lentivirus (10 ng, P24 measuring viral titer) in the presence of 8ug/ml polybrene, defined as day 0. On day 6, transduced cells were harvested by trypsinization and 1x10⁵ (cells) were seeded back onto mouse feeders (Mitomycin C treated, M0503, Sigma) in 6-well plates culturing with fibroblast medium. From day 7, the medium was switched into iPSC knockout medium and cells were fed daily with at least 2 ml iPSC knockout medium per well. iPSC colonies with round and compact morphology were visualized around 14 days post induction. After 3 or 4 weeks of reprogramming, the ES-like colonies were manually picked and grown on fresh mouse feeders to establish iPS clones. For feeder-free cultures of pluripotent cells, the iPSC and H9ES colonies were mechanically dissociated into small pieces and split onto VTN-N coated 4-well dishes in early passage. Then the iPSC and ES cells were maintained in E8 medium and routinely passaged with ReLeSR at later passage.

2.3. Quick validation with TRA-1-60 and CD44 live cell imaging

For quick validation of pluripotent cells, iPSC and ES cells were stained by TRA-1–60 (A25617, Molecular Probes) according to the instructions of Live Cell Imaging. Briefly, iPSC and H9ES cells were washed with PBS and incubated 30 mins at 37 °C by TRA-1–60 dye-conjugated antibodies with 1:50 dilution in E8 medium. After washing with FluoroBrite DMEM (A1896701, Molecular Probes), fluorescence of the cells was imaged within 30 mins. To continue culturing cells, the FluoroBrite DMEM medium was replaced by E8 medium.

MSCs were positive for CD44 and negative for TRA-1-60 markers. The iPSC-iMSC and H9ES-iMSC were stained by Live Cell Imaging Kit CD44 (A25527 Molecular Probes) and TRA-1–60 (A25617 Molecular Probes) for quick validation. The iPSC-iMSC and H9ES-iMSC in passage 3 (P3) with morphologically homogeneous population were seeded onto 4-well Chamber Slides (177437 Lab-Tek) at a density of 10,000 cell/cm². After 3 days culturing Live Cell Staining (CD44 TRA-1-60) was performed on MSCs as above procedure.

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2.4. Characterization of pluripotency by immunostaining

iPSC and H9ES cells were seeded onto 4-well Chamber Slides in E8 medium. When the cells were 60%-70% confluent, they were fixed by 4% paraformaldehyde for 30 mins. After three gentle washes with PBS, the fixed cells were penetrated with 0.3% Triton X-100 in PBS for 30 mins, followed by blocking with 5% donkey serum in PBS for 30 mins at room temperature. Then, cells were incubated with primary antibodies overnight at 4 °C against OCT4 (1:400), NANOG (1:200), SSEA-3 (1:200), SSEA-4 (1:200) and TRA-1–60 (1:200) individually. On the second day, cells were washed and incubated with secondary antibodies (1:500) diluted in 0.1% Triton X-100-PBS for 2 h without lights. Nuclei of cells were visualized with DAPI staining 10 mins. The cells were imaged with Leica fluorescence microscope (100X). The primary and secondary antibodies for characterization of pluripotency are shown in Supplemental Table 1.

2.5. Embryoid bodies (EBs) in vitro formation and differentiation

EBs formation and differentiation were performed by an optimized self-aggregated protocol. Briefly, the assay was initiated by seeding iPSC or H9ES cells onto low-attachment 24-well plates (Corning) by ReLeSR treatment and cells were cultured in suspension in E8 medium for 7 days. The EB medium was changed every other day. Then the mature EBs with aggregating morphology were harvested using 0.05% gelatin coated 4-wells Chamber Slides in high glucose DMEM medium supplemented with 10% FBS for spontaneous differentiation up to three weeks. The medium was replaced every 3 days. Differentiated cells were fixed by 4% PFA for 30 mins. The immunocytochemistry analysis of 3-germ layer of EB differentiation are listed in Supplemental Table 2.

2.6. Simple method for differentiating iPSC and H9ES cells into iPSC-iMSC and H9ES-iMSC

The iPSC and H9ES cells were adapted from cell co-cultured with mouse feeders onto VTN-N coated dishes and cultured in E8 medium before MSC derivation. A detailed procedure of iMSC generation from iPSC and H9ES cells can be found in our previous publication with minor modification (Zhou et al., 2018). Briefly, iPSC and H9ES cells were seeded onto VTN-N coated 6-well plates by ReLeSR in E8 medium to allow them to adhere for at least 2 days. At 60–70% confluency, pluripotent stem cells were washed with PBS and E8 medium was replaced by MSC low glucose growth medium. After around 2 weeks with medium switched every 2 days, the cells were passaged using 0.05% trypsin-EDTA (25300–062, Gibco) onto 0.1% gelatin coated T25 flasks. When cells were 80%-90% confluent (about 7 days), they were collected by 0.05% trypsin-EDTA and evenly seeded into new culture flasks. After 5 or 6 additional days of culturing in MSC low glucose growth medium, these derived intermediate cells were expanded at a density of 1x10(4) cells/cm², here defined as passage 0 (P0) of iPSC-iMSC and H9ES-iMSC. These fibroblast-like iPSC-iMSC and H9ES-iMSC were routinely split by trypsinization to be gradually homogenized for characterization and later experiments. BM-MSCs were passaged with a density of 6000 cells/cm² as reference cells.

2.7. Surface markers comparison in iPSC-iMSC, H9ES-iMSC and BM-MSC by FACS

The cell surface markers of iPSC-iMSC/H9ES-iMSC (P5) and BM-MSC (P6) were analyzed by flow cytometry (FACS). Antibodies against human cell surface antigens including CD11b, CD14, CD29, CD31, CD34, CD44, CD45, CD73, CD90, CD105 and HLA-DR (Zou et al., 2013) plus additional positive surface marker CD166 (12–1668, eBioscience) were used in this assay (Supplemental Table 2). When cells are 80–90% confluent, MSCs were trypsinized, washed with 2% FBS-PBS twice and re-suspended at concentration of 2x10(6) cells/ml. Then 100ul cell suspension aliquots (2x10(5) cells) were incubated with the conjugated antibodies for 30 mins at room temperature in the dark. Unstained cells were used as negative control. Then MSCs were washed with 2% FBS-PBS twice again, and fixed with 500ul 1% formaldehyde-PBS. For each sample, 10,000 events were recorded by FACS (LSRFortessa). Data was analyzed using FlowJo software. The positive ratios of surface MSC markers were normalized to negative control.

2.8. In vitro osteogenic, chondrogenic and adipogenic differentiation of MSCs

The iPSC-iMSC, H9ES-iMSC at passage 5 (P5) and BM-MSC at passage 6 (P6) were used for multipotent differentiation (osteogenesis, chondrogenesis and adipogenesis) with three replicates.

Osteogenic differentiation was performed according to the Osteogenesis Differentiation Kit (A10072-01, Gibco) instruction. Briefly, MSCs were cultured on 24-well plates at a certain seeding density (1x10(4) cells/cm² for iMSC and 6000 cells/cm² for BM-MSC) for 3 days in MSC low glucose growth medium. Medium was then replaced with osteogenesis differentiation medium to initialize differentiation. Cells in several wells with MSC low glucose growth medium were used as the undifferentiated group. Culture medium was changed every 3 days up to 3 weeks. Alkaline phosphatase (ALP) activities of cells were characterized by alkaline phosphatase staining (86R-1KT, Sigma) on 14 days post differentiation. Differentiated cells were also subjected to 2% Alizarin Red S (A5533, Sigma) staining (PH4.2) for calcium deposit identification following the manufacturer’s instructions after 3 weeks differentiation.

To induce chondrogenic differentiation, a micro-mass culture system was applied following the instruction of Chondrogenesis Differentiation Kit (A10071-01, Gibco). For the first step of chondrogenic differentiation, iMSCs and BM-MSCs were collected by trypsinization and suspended at a concentration of 1.6x10(7) cell/ml in MSC low glucose growth medium. Micro-mass of iMSC and BM-MSC were generated by seeding only 5ul droplets in the center of 96-multwell plates in humidified conditions for culturing 2 h. Then 150ul chondrogenesis differentiation medium for differentiation wells and MSC low glucose growth medium for control wells were separately supplemented onto top of the micro-mass cultivating. The primary chondrogenic pellets of MSCs were formed after one week of differentiation. The mature chondrogenic pellets were generated after 4 weeks differentiation, then embedded, sliced and dyed by toluidine blue staining for evaluation of extracellular chondrocyte matrix.

For adipogenic differentiation, the iMSCs (at a seeding density of 10,000 cell/cm²) and BM-MSCs (at a seeding density of 6000 cell /well) were cultured in 24-well plate according to instruction of Adipogenesis Differentiation Kit (A10070-01, Gibco). The cells were fed with adipogenesis differentiation medium or MSC low glucose growth medium every 3 days. After 3 weeks induction, both the differentiated and undifferentiated cells were fixed by 4% formaldehyde and stained with Oil Red O (Sigma-Aldrich) solution to validate the generation of lipid droplets.

2.9. In vivo cartilage defect repair using iMSCs

All the animal procedures were approved by the Animal Care and Use Committee of Shenzhen University. Eighteen male Sprague-Dawley (SD) rats (~250 g) were administered routine anesthesia. Then cartilage defect surgery was performed to make full-thickness cartilage defects (1.8 mm in diameter, 2 mm in depth) in the lateral femoral
condyle of the right knee of each rat. Subsequently, these rats were randomly divided into three groups (n = 6/group), and the mixture containing 1x10(6) cells was immediately injected into defected site as following groups: 2% hyaluronic acid (HA) gel (HA gel control), human iPSC-iMSC and HA gel mixture (iPSC-IMSC/HA gel), rat BM-MSC and HA gel mixture (rBM-MSG/HA gel). Three rats without cartilage defects were used as un-defect control. The animals were allowed to have free movement, and were sacrificed 12 weeks post operation. The femoral samples from the right knee of each rat were collected and fixed with 4% paraformaldehyde, followed by Safranin O staining and immunohistochemical staining of type II collagen (Col II).

2.10. RNA-sequencing for transcriptome profiling

During iMSC derivation from iPSC and H9ES cells, cell pellets with three replicates from each group on day 0, 7, 14, 21, 27 (P0) were collected and total RNA of cell pellets were extracted by RNAeasy Plus Mini Kit (74136, QIAGEN). Meanwhile total RNAs were also extracted from BM-MSG, iPSC-iMSC and H9ES-iMSC on P5 as control samples. Integrity of extracted RNAs was evaluated by RNA electrophoresis. At least 2.5ug bulk RNAs of each sample with a high RNA integrity were used for transcriptome sequencing in BGI, China. The cDNAs were reverse-transcribed from RNA samples. Then cDNA was PCR amplified, sheared, cleaned up using Beckman Coulter SPRSelect beads and quantified with Bioanalyzer High Sensitivity Kit. Sequencing libraries were constructed using MGIEasy RNA Library Prep Kit with barcoded adaptors for multiplexing libraries. The concentration of pooled libraries was determined by Bioanalyzer High Sensitivity Kit and then pooled libraries were sequenced with BGISEQ500.

2.11. Bioinformatic workflow for RNA sequencing data analyses

The quality of single end BGISEQ500 RNA-seq reads were checked by removing low quality reads, short reads, and residual adaptor sequences with SOAPoule software (Chen et al., 2018). Then the qualified reads were aligned into human gene transcripts (hg19) using bowtie2 software (Langmead and Salzberg, 2012). TPM (transcript per million) values, acquired by RSEM software (Li and Dewey, 2011), were used for statistical analyses of differential gene expression. Hierarchical clustering was applied to all detected genes via pvclust package (Suzuki et al., 2016). All experiments described in this study were conducted at least three replicates from each group on day 0, 7, 14, 21, 27 (P0). We next systematically analyzed the mesenchymal characteristics of these iMSCs by antigen profiling of six positive markers (CD29, CD44, CD73, CD90, CD105 and CD166) and six negative markers (CD11b, CD14, CD31, CD34, CD45 and HLA-DR) at both early and late passages (P5 and P10) by flow cytometry (Supplemental Fig. 2 and Supplemental Fig. 3). The bone marrow-derived mesenchymal stromal cells were used as a positive control and were analyzed for the expression of these 12 markers at passage 6 and passage 12. The majority of iMSCs (> 90%) were positive for all six mesenchymal positive markers and negative for all six lymphatic and endothelial markers (Supplemental Fig. 4), suggesting that the iMSCs generated with the feeder-free protocol possessed a high purity and mesenchymal characteristics. The positive rate of one mesenchymal marker, endoglin (ENG, CD105) which is a type I membrane glycoprotein, is lower in iMSCs (85–96%) than that in BM-MSCs (98–99%). This was consistent with what we had observed previously (Kang et al., 2015).

3. Results

3.1. Pluripotency characterization of human pluripotent stem cells

To achieve feeder-free derivation of induced mesenchymal stromal cells (iMSC), we firstly adapted our pluripotent stem cell culture system, which was previously based on MEF feeders and Knockout medium (Zou et al., 2013), to VTN-N and E8 medium (Zhou et al., 2018). We used two human pluripotent stem cell lines throughout the study: a human induced pluripotent stem cell line (iPSC) generated with lentiviral reprogramming as described previously (Zhou et al., 2017) and a human embryonic stem cell line (H9ES cell). The VTN-N-based feeder-free system could continuously support the proliferation of both lines and maintain stem cell-like morphologies (Supplemental Fig. 1A and B). We also confirmed the continuous expression of a pluripotency gene, TRA-1–60, by a live-cell imaging assay at early passage of both lines (Supplemental Fig. 1A and B). Immuno-fluorescence staining of pluripotency markers (TRA-1–60, OCT4, NANOG, SSEA3 and SSEA4) further validated the pluripotency of both lines under the feeder-free culture system (Supplemental Fig. 1A and B). Finally, embryoid body-mediated random differentiation indicated that both lines can give rise to ectoderm (beta-III tubulin, TUJ1), mesoderm (smooth muscle actin, SMA), and endoderm (Alpha-fetoprotein, AFP) (Supplemental Fig. 1C). These results taken together demonstrated that H9ES cell and iPSC line maintained their pluripotency under the feeder-free culture system and was subsequently used for following studies.

3.2. Feeder-free derivation of iMSCs

To investigate if iMSCs could be derived from human pluripotent stem cells cultured with the VTN-N feeder-free system, we adapted our previous protocol (Zou et al., 2013) and switched the E8 medium into MSC differentiation medium when the pluripotent stem cells reached approximately 60%–70% confluency. As indicated in Fig. 1A, the MSC differentiation medium was refreshed every 2 days. A significant change in morphology of the pluripotent stem cells was observed 7 days post differentiation (Fig. 1B, Day 7). After approximately 2 weeks of culture in MSC differentiation medium, spindle-shaped cells appeared (Fig. 1B, Day 14). From Day 14, a trypsin-based selection step was applied to facilitate the derivation of MSCs. We passaged the cells by trypsinization onto a 0.1% gelatin-coated flask at 1:1 ratio until Day 21 (Fig. 1B, Day 21). Cells were defined as passage 0 (P0) at Day 27. A homogeneous cell population with spindle-shape and MSC-like morphology were generated by three subsequent serial passaging (passage 3) with trypsinization when cells reach approximately 80% confluency (Fig. 1C), with a seeding density of 10,000 cells/cm² as our previous studies (Zou et al., 2013). Using live cell imaging, we firstly validated that the iMSCs derived from iPSCs and H9ES cells expressed the MSC marker phagocytic glycoprotein 1 (CD44) and were negative for the pluripotency marker TRA-1–60 (Fig. 1D).

3.3. Functional characterization of iMSCs

We next systematically analyzed the mesenchymal characteristics of these iMSCs by antigen profiling of six positive markers (CD29, CD44, CD73, CD90, CD105 and CD166) and six negative markers (CD11b, CD14, CD31, CD34, CD45 and HLA-DR) at both early and late passages (P5 and P10) by flow cytometry (Supplemental Fig. 2 and Supplemental Fig. 3). The bone marrow-derived mesenchymal stromal cells were used as a positive control and were analyzed for the expression of these 12 markers at passage 6 and passage 12. The majority of iMSCs (> 90%) were positive for all six mesenchymal positive markers and negative for all six lymphatic and endothelial markers (Supplemental Fig. 4), suggesting that the iMSCs generated with the feeder-free protocol possessed a high purity and mesenchymal characteristics. The positive rate of one mesenchymal marker, endoglin (ENG, CD105) which is a type I membrane glycoprotein, is lower in iMSCs (85–96%) than that in BM-MSCs (98–99%). This was consistent with what we had observed previously (Kang et al., 2015).

According to minimal criteria for MSCs properties (Dominici et al., 2006), the capability of tri-lineage differentiation in vitro is one "gold standard" for characterizing MSCs functions. Thus, we performed osteogenic, chondrogenic and adipogenic differentiation for iMSCs, and used BM-MSCs as controls. The osteogenic capacity was evaluated by seeding iMSCs (P6, with a density of 10,000 cell/cm²) and BM-MSCs (P8, with a density of 6,000 cell/cm²) onto 0.1% gelatin coated dishes and differentiated with osteogenic differentiation medium (detail process referred to MATERIALS AND METHODS). Cells cultured in MSC
were used as negative control. Positive staining for ALP was observed for both iMSCs and BM-MSCs after 14 days in the osteogenic differentiation medium (Fig. 2a–f). Calcium deposition was robustly detected by Alizarin Red S staining of extracellular calcium on 21 days post differentiation (Fig. 2g–l). For the chondrogenic differentiation, we used a self-aggregating protocol in 96-well plates in micro-mass cultures (detail process referred to MATERIALS AND METHODS). We successfully generated aggregates from both iMSC lines, but not with the BM-MSCs. After 4 weeks of differentiation using a standard chondrogenic medium, the chondrogenic differentiation cell aggregates were analyzed for extracellular chondrocyte matrix by toluidine blue staining (Fig. 2m–n). Strong staining by toluidine blue was observed for iMSCs derived from iPSC and H9ES cells. Consistent with what we observed previously (Kang et al., 2015), the iMSCs generated using the
feeder-free method also possessed modest adipogenic capacity compared with BM-MSCs by Oil Red O staining of small lipid droplets on day 21 (data not shown). Taken together, we here described and characterized an improved method for the generation of functional iMSCs, which possesses potent osteogenic and chondrogenic capacities.

To assess the regenerative properties of our iMSC, we generated a rat cartilage defect model, then transplanted the iPSC-iMSC/Hyaluronic acid (HA) gel mixture into these cartilage defect rats (Supplemental Fig. 5A), with rat BM-MSC (rBM-MSC)/HA gel mixture transplantation as positive control and HA gel alone injection as negative control. 12 weeks post-surgery, we observed the cartilage defects were markedly more filled after iPSC-iMSC or rBM-MSC implantation at the macroscopic level in comparison to HA gel control group (Supplemental Fig. 5B. a-d). In iPSC-iMSC/HA gel group, the majority of repair tissue was homogeneous and positive with Safranin O staining compared to HA gel alone group, which demonstrated significant formation of cartilage after iPSC-iMSC implantation. As expected, the repaired tissue recovered from the defects with more cartilage formation and obvious cartilage integration in rBM-MSC/HA gel group (Supplemental Fig. 5B. e-h). We also observed strong expression of Col II in the immunohistochemistry staining of the iPSC-iMSC/HA gel group, with a similar expression level as that of rBM-MSC/HA gel group. In contrast, the Col II expression was rarely detectable in most repair tissues of HA gel control group (Supplemental Fig. 5B. i-l). Overall, although the repair did not match the native cartilage in the un-defect group, the cartilage defects in iPSC-iMSC implantation were remarkably reduced, almost reaching the repair level of rBM-MSC treatment group. In addition, we did not observe tumor formation in these rats with normal immune systems during iMSC implantation and cartilage defect repair. Collectively, our iMSCs possessed strong cartilage regeneration capability similar to BM-MSC and have the potential for therapeutic uses.

3.4. The genome-wide transcriptome reference of iMSCs

One important resource for MSC research that has not been fully investigated is a genome-wide transcriptome reference of MSCs. A number of studies have investigated the transcriptome of BM-MSCs, pluripotent stem cells and/or fibroblasts separately (Yi et al., 2019; Nguyen et al., 2018; Kuno et al., 2018). However, there is still a lack of the transcriptome reference which systematically combines the genome-wide transcriptome in human
were also included as controls. Using the DNA nanoball sequencing transcriptome analysis (Fig. 3). Total RNA from BM-MSCs and fibroblasts. After the pluripotent state, intermediate state and mature iMSCs (passage 5) for feeder-free iMSC derivation method, we harvested total RNA from cells at BM-MSCs and fibroblasts. With the establishment and validation of the pluripotent stem cells, intermediate states during iMSC derivation, iMSCs, and fibroblasts were differentially expressed (fold change > 2, FDR p-value < 0.05) during the differentiation of pluripotent stem cells to iMSCs. Considering that the differentiation rate might differ between the H9ES cells and the iPSC line, the comparison was carried out separately for these two lines (Fig. 5A-E, iPSC-D7), MSCs (H9ES-iMSC, iPSC-iMSC and BM-MSC), intermediate cells in iMSC derivation (D7, 14, 21, P0); 3 normal human dermal fibroblasts (NHDF) lines were used as control.

Fig. 3. The genome-wide transcriptome analysis of the feeder-free iMSC derivation. (A) Schematic representation of cell collection for RNA seq in iMSC derivation from H9ES cells and iPSC on Day 0, 7, 14, 21, 27 (passage 0, P0) and P5. (B) The number of expressed genes (TPM > 1) from cells in iMSC derivation detected on Day 0, 7, 14, 21, 27 (P0) and P5.

Fig. 4. Unsupervised clustering for global transcriptome similarity. Three main clusters were identified, including the pluripotent stem cells (H9ES cells, iPSC, iPSC-D7), MSCs (H9ES-iMSC, iPSC-iMSC and BM-MSC), intermediate cells in iMSC derivation (D7, 14, 21, P0); 3 normal human dermal fibroblasts (NHDF) lines were used as control.

3.5. iMSCs are transcriptomically similar to BM-MSCs

To investigate the transcriptome similarity between iMSCs and other cell types, we performed an unsupervised hierarchical clustering analysis for all samples. Three clearly separated clusters were obtained: a pluripotent stem cell cluster, a MSC cluster and an intermediate cell cluster (Fig. 4). We noted that the day 7 differentiated cells from the iPSCs were still clustered with the pluripotent stem cells, suggesting that the differentiation rate of this iPSC line is slower than the H9ES cells. Consistent with our previous observation (Zou et al., 2013), early passage (P0) of iMSCs were still immature, which clustered with other intermediate states of cells and the fibroblasts (NHDF). Most importantly, the two iMSC lines derived from iPSC and H9ES cells were clustered with the BM-MSCs, suggesting that the iMSCs generated with the feeder-free approach recapitulate the global transcriptome profile of BM-MSCs.

3.6. Extracellular matrix structure and organisation are important for iMSC derivation

To identify what pathway regulates iMSC derivation, we performed pair-wise comparison with DESeq2 (Love et al., 2014) to identify genes that were differentially expressed (fold change > 2, FDR p-value < 0.05) during the differentiation of pluripotent stem cells to iMSCs. Considering that the differentiation rate might differ between the H9ES cells and the iPSC line, the comparison was carried out separately for these two lines (Fig. 5A-E, H9ES cells and Fig. 6A-E, iPSC). We observed significant down regulation of the pluripotency genes and up regulation of germ-layer commitment and differentiation genes during the first two weeks (Fig. 7). The loss of pluripotency and commitment to differentiation is faster in the H9ES cells as compared to the iPSC line. This was observed by a higher number of differentially expressed genes at D7 (Fig. 5F and 6F) and the higher degree of down regulation of pluripotency genes such as POU5F1, DNM3T3B, TDGF1 in the H9ES cells (Fig. 7). Another wave of high transcriptional change occurred in the earlier passages of the iMSCs, from immature to mature iMSCs. We performed gene-set enrichment analysis for all differentially expressed genes (Fig. 5G-K and Fig. 6G-K). Among a great number of pathways involving the differentiation and maturation of iMSCs, one pathway that has consistently appeared as the top pathway was extracellular matrix structure and organization, including genes such as TGFBI, SERPINE1, CTGF, COLA1 and ECM1 (Supplemental Fig. 6). The microenvironmental niches are of particular importance for proliferation and maintenance of stem cells. Our finding of the extracellular structure and matrix organization for mesenchymal stromal cells was highly consistent with many previous observations. For instance, down regulation of the TGFβ-induced gene product-h3 (TGFBI) was found in MSCs from patients of osteoarthritis and is related to the impair regenerative potential of MSCs (Ruiz et al., 2019). A recent study further showed that the TGFBI secreted via extracellular vesicles of mesenchymal
stromal cells could ameliorate osteoarthritis (Ruiz et al., 2020). As observed previously by us and also demonstrated here, the iMSC retains robust osteogenic and chondrogenic capacities but less adipogenic potential. We compared the iMSCs to BM-MSCs and found that the extracellular matrix structure and organization were significantly different between the two sources of MSCs (Supplemental Fig. 7). Our results taken together suggest that extracellular matrix structure and organization are important for iMSC derivation and functions.

### 3.7. Identification of MSC-enriched markers

It has been observed that all current MSC-specific markers are also expressed in fibroblasts (Denu et al., 2016). The six commonly used MSC-positive (CD29, CD44, CD73, CD90, CD105 and CD166) and MSC-negative (CD11b, CD14, CD31, CD34, CD45 and HLA-DR) markers were also positive and negative for fibroblasts, respectively (Fig. 8A). However, the expression levels of some of the positive markers were significantly different between MSCs and fibroblasts. For instance, the expression of NT5E and ALCAM are higher in MSCs than fibroblasts, whereas THY1 expression is lower in MSCs than fibroblasts. Based on this observation, it is recommended to include the criteria of gene expression level for isolation and enrichment of MSCs.

We next applied the classification of enrichment (Thul et al., 2017) to identify genes which have enriched expressions in MSCs as compared to pluripotent stem cells, intermediate cells and fibroblasts. The following criteria were used: (i) The mean gene expression level (TPM) of MSCs (iMSCs and BM-MSCs) was > 5; (ii) The mean gene expression in MSCs was 3-fold higher than the mean gene expression in non-MSC cells (iPSC, H9ES cells, intermediate cells (D7, D14, D21 and P0), fibroblasts); (iii) The mean gene expression in MSCs was 3-fold higher than that in fibroblasts; and (iv) The P value was < 0.05 (MSCs vs. non-MSCs). Using this classification, we identified 52 genes that significantly distinguish MSCs from other cell types (Fig. 8B). For example, six genes: fibronectin 1 (FN1, 8.1 folds), transforming growth factor beta induced (TGFBI, 10.5 folds), transgelin (TAGLN, 3.9 folds), serpin E-family member 1 (SERPINE1, 19.5 folds), insulin-like growth factor binding protein 7 (IGFBP7, 4.4 folds) and peristin (POSTN, 4.4 folds) were highly expressed in MSCs (mean TPM > 1584) compared to other cell types. Several of these genes have been previously found to be important for the multipotency of MSCs. IGFBP7 is a low-affinity IGF binder and has been found to regulate the osteogenic differentiation of MSCs (Zhang et al., 2018). It was interesting to observe that the FN1 was expressed in all cells, but at higher level in MSCs. This observation was consistent with the previous finding that FN1 regulates MSC migration (Veevers-Lowe et al., 2011) and our finding that extracellular matrix organization is important for MSCs. And lastly and most importantly, our dataset identified three genes that were highly expressed in MSCs than fibroblasts; EGF-like repeats and discoidin domains 3 (EDIL3, 77.8 folds), ankyrin repeat domain 1 (ANKRD1, 198.2 folds) and EPH receptor A5 (EPHA5, 377.6 folds). EDIL3 is an extracellular matrix protein that inhibits inflammatory bone destruction and regulates osteoblast differentiation (Oh et al., 2017). Likewise, Ankyrin repeat domain 1 protein (ANKRD1) is a transcriptional repressor of matrix metalloproteinase (MMP) 13, a mammalian collagenase capable of degrading collagens (Almodovar-Garcia et al., 2014; Li et al., 2017). Both EDIL3 and ANKRD1 play crucial roles in epithelial to mesenchymal transition regulation (Xia et al., 2015; Takahashi et al., 2018). Although the functional role of EPH receptor A5 for MSCs still remains to be fully explored, the higher expression of EPHA5 might be related to the immune suppressive function of MSCs (Nguyen et al., 2013).

To further identify the potential genes involved in MSC commitment, we applied time course analysis based on the RNA sequencing.

![Fig. 5. Comparison of gene differential expression and pathways in H9ES-iMSC derivation between different timepoints. (A-E) Differentially expressed genes were compared in H9ES-iMSC derivation in different timepoints (fold changes > 2, FDR p. value < 0.05). (F) Total un-regulated genes and down-regulated genes in H9ES-iMSC derivation in different timepoints. (G-K) Analysis of pathways involved in the differentiation and maturation of H9ES-iMSC in different timepoints. Different timepoints: D7 vs. D0, D14 vs. D7, D21 vs. D14, P0 (D27) vs. D21, P5 vs. P0.](image-url)
Using an unbiased clustering method (Futschik and Carlisle, 2005), we identified 12 clusters of genes that displayed a coherently staged-specific expression pattern (data not shown). Most importantly, we identified that genes in Cluster1 have shown a consistent increase in expression during the derivation of MSCs from H9ES cells and iPSC (H9ES-C1 and iPSC-C1) (Supplemental Fig. 8A). In these two clusters, 440 genes were identified to be commonly expressed in H9ES-iMSC and iPSC-iMSC derivation (total 764 genes and 969 genes were clustered separately) (Supplemental Fig. 8B). Further gene ontology analysis of genes in these two clusters revealed that focal adhesion and extracellular exosome pathways were significantly enriched in iMSC derivation from H9ES cells and iPSC (Supplemental Fig. 8C).

4. Discussion

In this study, we have successfully developed a feeder-free method with one-step medium switch to efficiently differentiate pluripotent iPSC and H9ES cells into multipotent iMSCs. This method excluded the risk of feeder cell contamination and provided the possibility for investigating the dynamic changes of gene expression during pluripotent stem cells differentiating into iMSCs. Meanwhile this method is easier (one-step medium switch) compared to FACS sorting (Lian et al., 2010) and more cost-effective without additional small molecules (Zhao et al., 2015; Chen et al., 2012). A recent report allowed large-scale generation of MSCs from human ESCs, however, hESCs needed to be cultured as spheroids in 3D systems and MSCs generation still needs additional small molecules in medium (Yan et al., 2018). During the entire differentiation process of our method, selective cell death was observed in the iPSCs and H9ES cells after switching to low glucose MSC medium and the gradual morphology change was observed for the remaining survived cells, from flat monolayer distinctive cobbled colony to spindle-shaped fibroblast-like cells. For this protocol, a serial expansion by trypsinization is crucial for MSC derivation and purity enrichment.

Consistent with our previous results (Zou et al., 2013; Kang et al., 2015), the iMSCs generated by this new method had similar immunophenotype as BM-MSCs. The iMSCs lost pluripotent markers (such as TRA-1-60, NANOG and OCT4), whereas they obtained typical mesenchymal surface markers (positive for CD29, CD44, CD73, CD90, CD105 and CD166; negative for CD11b, CD14, CD31, CD34, CD45 and HLA-DR). We observed that the iMSC proliferation capacity and immune phenotypes decreased after passage 15 (data not shown), it is thus recommended to use iMSCs between passage 3 and passage 10 for functional and translational studies.

Tri-lineage differentiation of the iMSCs generated with the current feeder-free method showed that the iMSCs possessed potent osteogenesis and chondrogenesis capacities with less adipogenicity, similar to our previous iMSCs generated using the feeder-based system (Kang et al., 2015, 2014) and other publications (Chen et al., 2012). Early studies have shown a 7-day treatment of stem cell-derived MSCs with high levels of glucose (25 mmol/L) before adipogenic differentiation can enhance adipogenesis through the Wnt/protein kinase C non-canonical pathway (Keats and Khan, 2012; Keats et al., 2014). We have
The scientific community can benefit from using this reference, we incorporate it into the existing data resource of iMSCs. To ensure that a broader range of users can benefit and promote the mesenchymal stromal cell research society, we present here a highly rich resource and preliminary data on genes and pathways that might play an important role in MSC lineage commitment. We acknowledge that more studies are required to functionally validate the identified genes and pathways. RNA sequencing with the NGS platform is a very powerful technology, which allows deep analysis of the whole transcriptome from any tissue through detecting known and unknown transcripts and quantifying a large number of genes. Meanwhile, to overcome the lack of easily accessible and comparative transcriptome reference for mesenchymal stromal cells, in this study we generated an NGS-based transcriptome reference including gene expression profile in pluripotent stem cells, intermediate cells during iMSC derivation, mature iMSCs, BM-MSCs and fibroblasts. A recent report showed a multi-omics study of the differentiation of human ESC into MSCs by profiling the transcriptomes, proteome and phosphoproteome. The researchers determined a set of high confidence transcription factors, kinases and phosphatases, as well as non-coding transcripts, in which the data resource would be useful for the study of MSC differentiation from human ESC (Billing et al., 2019). Comparatively, our study has systematically compared the transcriptome of iMSCs derived from iPSC and H9ES cells, which would provide a promising iMSC transcriptome reference supplementary to the existing data resource of iMSCs. To ensure that a broader scientific community can benefit from using this reference, we incorporate the data into a defined excel database which allows users to explore the data easily.

Compared to iPSC, we observed higher differentiation kinetic in H9ES cells (Fig. 5F and 6F), in which more differentially expressed genes at D7 were detected in H9ES cells. Furthermore, the down-regulation of pluripotency genes and the differentiation rate is faster in the H9ES cells (Fig. 4). Although some iPSC lines are indistinguishable from ES clones in their gene expression, iPSCs generally differ from ES cells in their DNA methylation and differentiation ability (Yamanaka, 2012). A number of studies have already shown that iPSC lines retain an epigenetic memory of their cell type of origin (such as fibroblasts, keratinocytes, and peripheral blood mononuclear cells). The epigenetic signature in iPSCs is able to persist even after extensive passaging (Kim et al., 2010; Ohl et al., 2011). The epigenetic memory (such as methylation patterns) derived from different somatic cells does indeed influence the differentiation capacity of iPSC. Therefore, it is interesting to investigate the transcriptome change among multiple IMSC lines derived from iPSC reprogrammed from different somatic cells to further understand the mechanism of iPSC differentiation into IMSCs.

We have observed that extracellular matrix structure and organization pathway was highly enriched during the differentiation of H9ES cells and iPSC into IMSCs after the gene-set enrichment analysis (Fig. 5G-K). It will be very useful to analyze the differential expression of key proteins in extracellular matrix structure and organization pathway during iMSC derivation from H9ES cells and iPSC (between D14 to D21 and D21 to P0 for H9ES cells and between D7 to D14 and D14 to D21 for iPSC line). This can facilitate the understanding of the protein composition of extracellular matrix structure and organization pathway.

In the unsupervised hierarchical clustering analysis for the global transcriptome of IMSCs derived from H9ES cells and iPSC, we obtained 3 well-separated clusters based on the transcriptome similarity (Fig. 4). For the intermediate cell cluster, analysis of the expressions of osteogenic genes (RUNX2, ALP, COL1A1 and OC) and chondrogenic genes (Collagen type II and Collagen type XI) in different time points (D7, D14, and D21) would be useful to evaluate the functional properties of the intermediate cell populations and to identify when iMSC could acquire osteogenic and chondrogenic differentiation potential. In addition, RNA sequencing data showed that MSC-specific markers were also positively expressed in fibroblasts (Fig. 8A), which was consistent with our FACS analysis of MSC-specific markers on fibroblasts (data not shown).

Recently, scRNA-seq technology has dramatically developed to reveal the heterogeneity of cells (Tabula Muris, 2018). Mesenchymal stromal cells have been considered to be a heterogeneous population. A recent study analyzed 361 single MSCs from human umbilical cords and revealed limited heterogeneity (Huang et al., 2019). However, the power of cells used in this study was too low to draw a significant conclusion on MSC heterogeneity. Another study analyzed 6,176 MSCs from human umbilical cords and identified clusters of MSCs which exhibit unique functions (Sun et al., 2019). Further studies that greatly benefit and promote the mesenchymal stromal cell research society and translational research will be generating an MSC atlas for mesenchymal stromal cells isolated from different tissues of healthy and pathological conditions.

Taken together, we present here a highly rich resource and preliminary data on genes and pathways that might play an important role for MSC lineage commitment. We acknowledge that more studies are required to functionally validate the identified genes and pathways. This study provided a first full list of genes (both known and novel ones) which have highly enriched expressions in IMSCs and the transcriptome resource of iMSC derivation from PSCs will be highly valuable for the mesenchymal stromal cell research society.

5. Conclusion

We have optimized a feeder-free method for the generation of iMSCs from human pluripotent stem cells, in which it is able to generate IMSC

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**Fig. 7.** Comparison of pluripotency and germ-layer commitment genes in H9ES-iMSC and iPSC-iMSC derivation. Heatmap showed gene expression (log2 fold change as compared to mean expression at the pluripotency level, day 0) in different timepoints: D0, D7, D14, D21, D27 (P0), PS. BM-MSC was used as reference. Genes include key pluripotency genes, ectoderm differentiation genes, mesoderm differentiation genes and endoderm differentiation genes.
at large scale in an easy and effective way. Meanwhile, we provided important transcriptome references for pluripotent stem cells, intermediate differentiated cells, MSCs and fibroblasts by RNA-sequencing. These transcriptome resources would facilitate the understanding of MSCs differentiation from human pluripotent stem cells and MSC functions in regenerative medicine.

CRediT authorship contribution statement


Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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