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Common Expression of Stemness Molecular Markers and Early Cardiac Transcription Factors in Human Wharton's Jelly-Derived Mesenchymal Stem Cells and Embryonic Stem Cells

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At present, there are still significant barriers that impede the clinical use of hESCs and iPS cells, including ethics, immunorejection, tumorigenesis from hESCs, and teratoma formation from iPS cells. It is therefore necessary to search for alternative sources of stem cells. WJ-MSCs originate from embryonic epiblasts and possess properties intermediate between hESCs and adult stem cells. However, the stemness properties of molecules in WJ-MSCs remain unclear compared to those of hESCs. In the present study, we isolated WJ-MSCs by a nonenzymatic method. Further, using microarray analysis by Affymetrix GeneChip and functional network analyses, we determined the degree of expression of stemness genes exhibited by the Human Stem Cell Pluripotency array. We also defined a wide range of stem cell gene expression in the WJ-MSCs in comparison with hESCs. At the same time, the definitive markers of early cardiac precursor cells and more committed progenitors were further characterized in WJ-MSCs. Our results demonstrated for the first time that WJ-MSCs had significant expression of undifferentiated human embryonic stem cell core markers, such as SOX2, NANOG, LIN28, SSEA1, SSEA3, SSEA4, KLF4, c-MYC, CRIPTO, and REX1, with a relatively lower level of expression than in hESCs. We also found WJ-MSCs have high expression of early cardiac transcription factors, such as Flk-1, Isl-1, and Nkx2.5. Functional analysis revealed signature genes of WJ-MSCs with specific roles involved in immune, cytoskeletal, and chemokine regulation, cell adhesion, and cell signaling. Our study indicated that there is a significant overlap between the stemness genes expressed by hESCs and WJ-MSCs. WJ-MSCs harbor a true stem cell population and are promising cells for stem cell-based therapies.

Key words: Stemness genes; Early cardiac transcription factors;
Wharton's jelly-derived mesenchymal stem cells (WJ-MSCs); Human embryonic stem cells (hESCs)

INTRODUCTION

Ischemic heart failure after myocardial infarction is a leading cause of death worldwide. The progressive and irreversible loss of cardiac myocytes due to myocardial infarction is a feature of ischemic cardiomyopathy despite current therapies such as pharmacological treatment, catheter-based therapy, and surgical interventions (44,55). Recent experimental and clinical studies, however, have opened a new exciting potential therapeutic strategy involving the transplantation of stem cells into the injured heart (39,41). Many potential cell sources can be utilized, including skeletal myoblasts (31,43), mesenchymal stem cells (MSCs) (33,42,52), and endothelial progenitor cells (4,21). It has been demonstrated that MSCs have the potential to differentiate into cardiomyocytes both in vitro and in vivo (28,40,49,57,64). A number of clinical trials have been performed using autologous bone marrow-derived MSCs. However, the results of these trials have been unsatisfactory because of the poor efficiency of differentiation into mature myocardium and a low number of MSCs in older patients with coronary heart disease (2,3,17,27,31,32,47,59). The ultimate goals of cell therapy are myocardial regeneration and neovascularization, leading to clinical improvement without severe adverse effects. To this end, it is necessary to search for alternative sources of stem cells that have greater proliferation and differentiation potential.

Pluripotent stem cells derived from the inner cell mass of early stage embryos have provided a prototype for multilineage repair. Currently, human embryonic stem cells

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(hESCs) have been successfully converted in vitro into functional cardiomyocytes, and transfer of such cells into ischemic animal models has shown successful engraftment (22,24,62,63). However, hESC-derived tissue application in the human is fraught with the problems of ethics, immunorejection, and tumorigenesis. These considerations, along with practical limitations, have precluded adoption of embryonic stem cell platforms (38). On the other hand, the recent landmark discovery that mouse and human somatic cells can be reprogrammed to a ground state of pluripotency by ectopic expression of only a few defined transcription factors offers a novel fascinating route to patient-specific pluripotent cells, without the technical and ethical limitations of somatic cell nuclear transfer (29,30). However, the therapeutic value of reprogramming remains largely unknown because of the problem of teratoma formation and long-term stability of induced pluripotent stem cell (iPSC)-derived tissues. Therefore, iPSCs still have significant problems that impede their adaptation into the clinic (26).

The human umbilical cord (UC) is an extraembryonic formation that constitutes the essential link between the mother and her fetus during pregnancy. UC is composed of two arteries and a vein, all of which are surrounded by a unique connective tissue stroma, namely, Wharton's jelly (WJ). WJ originates from extraembryonic and/or embryonic mesoderm at day 13 of embryonic development (45). WJ surrounds embryonic blood island cells during their migration to the aorta-gonad mesonephros region from the yolk sac region prior to day E 10.5 (8,45,53). Several reports have shown that stem cells with pluripotent differentiation potential, extraembryonic mesoderm stem cells, can be isolated from WJ in humans. These cells fulfill most criteria for MSCs, such as adherence to plastic, expression of characteristic surface markers, and differentiation into cells of mesenchymal origin, including bone, cartilage, adipose tissue, and cardiomyocytes (6,12,19,46,48,53,56). In contrast to bone marrow MSCs, Wharton's jelly mesenchymal stem cells (WJ-MSCs) have greater expansion capability, faster growth in vitro, and may synthesize more and different cytokines (11,18,60). It has been found that matrix stem cells derived from porcine umbilical cord or human Wharton jelly express markers characterized in embryonic stem cells, such as the transcription factors Nanog, octamer binding transcription factor-4 (Oct-4), sex determining region Y box 2 (Sox-2), and guanine-adenine-thymine-adenine binding protein 4 (GATA-4), GATA-5, and GATA-6 (10,37). A more recent study showed that WJ-MSCs, functioning as human extraembryonic mesoderm stem cells, express the key early cardiac transcription factors GATA-4, GATA-5, and GATA-6 (25). Especially, it was also demonstrated that WJ-MSCs express connexin-43 of intercellular gap junctions (56). Moreover, WJ-MSCs express genes with immune regulatory properties, which are immunoprivileged (25,58). Therefore, WJ-MSCs may represent a rich source of primitive cells derived from the extraembryonic tissue and may be a promising cellular source for cardiac stem cell-based therapy (3,27,38,61).

As described above, although it has been demonstrated that WJ-MSCs express many genes like ESCs that are markers of stemness, the difference in molecular properties between WJ-MSCs and hESC has yet to be established. Even more, the similarities and differences in the global gene profiles between WJ-MSCs and hESCs, as well as which common molecules are shared by them, are still not reported. So in this study, first we examined and compared the global gene expression profiles, and asked whether WJ-MSCs share the expression of a set of core stem marker genes with hESCs by using the Human Stem Cell Pluripotency array. Second, we subcategorized them as signature gene, early cardiac transcription factor, immune property gene, and growth factor and receptor genes, in order to identify major genomic differences and unique biological markers specific to the target cell population compared with human embryonic stem cells by using the Rhesus Affymetrix Gene Chip.

MATERIALS AND METHODS

Tissue Samples and Cellular Isolation Protocol

The work was conducted following the approval of the Navy General Hospital Ethical Review Board. With the consent of the parents, one fresh human female umbilical cord was obtained from a full-term birth with no complications throughout the pregnancy by caesarian section, aseptically stored in sterile saline, and processed within 6 h from partum to obtain the umbilical cord. After removal of blood vessels, the abundant extracellular matrix of Wharton's jelly, which is a mucous tissue continuum from the subamnion to the perivascular region, was scraped off with a scalpel, finely cut, rinsed in sterile phosphate-buffered saline [PBS, Hyclone, Logan, UT, USA; composition (in mM): 140 NaCl; 2 KCl; 1.5 KH_2PO_4; 15 Na_2HPO_4], and transported to the laboratory at room temperature. The Wharton's jelly was then serially cut in a cross-sectional manner, and four to five explants of the matrix, ranging from 1 to 2 cm, were placed in 100-mm tissue culture dishes (Corning, Corning, NY, USA) with 2–3 ml of culture medium [Dulbecco's modified Eagle's medium-F12 (DMEM-F12) low-glucose (Sigma-Aldrich, St. Louis, MO, USA), supplemented with 10% fetal bovine serum (FBS; PAA, Linz, Austria), 1× nonessential amino acids (Sigma), 1× antibiotics–antimycotics (Gibco, Gaithersburg, MD, USA), and 2 mM L-glutamine (Sigma)]. The isolation method made no use of proteases to detach cells from the embedding matrix. Therefore, based on the "mesenchymal" migratory capability of cells, cord Wharton's jelly fragments were left in the culture medium for 14 days, with medium change every second day. Cellular exit from the

cord Wharton's jelly and attachment to the plastic surface of the tissue culture slide was monitored by phase-contrast microscopy. Finally, after 15 days of culture, the remnants of the cord Wharton's jelly fragments were removed from the wells, and cells that attached to wells were cultured until they reached confluence. A single human female ESC line was obtained from the Shanghai Institutes for Biological Sciences Chinese Academy of Sciences (Shanghai, China). This cell lineage is not commercially available. In brief, the female ESCs were maintained on a mitotically inactivated (mitomycin C) mouse embryonic fibroblast (Shanghai Institutes for Biological Sciences) feeder layer in DMEM-F12 supplemented with 20% knockout serum replacer, 0.1 mmol/L nonessential amino acids, 1 mmol/L L-glutamine (all from Invitrogen, Carlsbad, CA, USA), 0.1 mmol/L β-mercaptoethanol (Sigma), and 4 ng/ml human recombinant basic fibroblast growth factor (Invitrogen).

Cell Culturing and Passaging

After reaching confluence, primary cells were subcultured routinely in culture medium. Cellular detachment from tissue culture dishes was performed using accutase (Invitrogen) instead of standard trypsin solution. Primary populations of WJ-MSCs were cultivated for up to three to six passages. For long-term storage, WJ-MSCs were also cryopreserved in a medium consisting of 93% FBS and 7% dimethyl sulfoxide (Sigma-Aldrich Inc.). For experiments, cells at passage 3 were used in order to ensure the maintenance of marker expression during cell doubling.

Immunophenotype Analysis

Immunophenotyping of the expanded WJ-MSCs was examined using flow cytometry. The adherent cells were washed with PBS and detached by incubating with trypsin/ethylenediaminetetraacetic acid (EDTA) (Hyclone) for 3 min at 37°C. The harvested cells were washed using staining buffer containing 4% FBS and 0.1% azide (Sigma) in PBS, and approximately $0.05–0.1 \times 10^6$ cells per tube was used for cell surface antigen expression studies. The cells were stained with phycoerythrin/fluorescein (FITC)-labeled monoclonal antibodies against human cluster of differentiation 44 (CD44), CD90, CD105, CD73, CD31, CD45, CD34, CD3, CD4, CD8, CD80, CD86, human leukocyte antigen (HLA)-ABC, and HLA-DR (Beijing Boiynthesis Biotechnology Co, Beijing, China) for 45 min at room temperature. The stained cells were run on a flow cytometer (FACSCalibur, Becton-Dickinson, Franklin Lakes, NJ, USA) equipped with a 488-nm argon laser to confirm WJ-MSC purity. Approximately 10,000 events were acquired and analyzed using the CellQuest software (Becton Dickinson). For viability determination, the cells were stained with 7-amino actinomycin D (7-AAD; BD Pharmingen, USA) and acquired on a flow cytometer. The results were analyzed using WIN MDI v 2.8 software (Becton Dickinson).

Differentiation Studies

Osteogenic Potential. For osteogenic differentiation, the third-passage WJ-MSCs were plated in a six-well chamber slide (Corning) with a count of 3,000–10,000 cells per well. After plating the cells in DMEM-F12 supplemented with 10% FBS or 24 h, osteogenesis was induced by replacing with differentiation medium [DMEM-F12 supplemented with 10% FBS, 0.1 μM dexamethasone (Sigma-Aldrich), 10 mM β-glycerophosphate (Sigma-Aldrich), and 50 μM ascorbic acid (Sigma-Aldrich)]. The differentiation medium was changed every 2 to 3 days. After 21 days of differentiation, the cells were checked for calcium deposition with the Von Kossa technique (Sigma).

Chondrogenic Potential. For chondrogenic differentiation, third-passage WJ-MSCs were harvested and differentiated into chondrocytes in pellet cultures at a density of $0.25–0.5 \times 10^6$ in a polypropylene tube (Corning) containing chondrogenic differentiation medium, which was DMEM-F12 supplemented with 0.1 μM dexamethasone (Sigma-Aldrich), 10 mM β-glycerophosphate (Sigma-Aldrich), and 50 μM ascorbic acid (Sigma-Aldrich). The tubes were incubated at 37°C in a 5% CO_2 incubator. The cells grew as pellets, which were fed with fresh chondrogenic medium every 2 to 3 days. Chondrogenic pellets were harvested after 14 to 28 days in culture. The harvested chondrogenic pellets were fixed with 10% formalin (Sigma-Aldrich) and paraffin-embedded for histological processing. Thin sections, approximately 4–10 μm, were also stained with Safranin O (Sigma-Aldrich).

Adipogenic Potential. For adipogenic differentiation, third-passage WJ-MSCs were plated in a six-well chamber slide with a count of 3,000–10,000 cells per well. Cells were incubated in DMEM-F12 supplemented with 10% FBS, 1 μM dexamethasone, 0.5 mM methylisobutylxanthine, 10 μg/ml insulin, and 100 μM indomethacin for 3 weeks and assessed by Oil Red O staining (Sigma-Aldrich). All inducing agents were procured from Sigma-Aldrich.

Cardiomyogenic Potential. The differentiation of cardiomyogenic (CMG) cells from WJ-MSCs was performed. The third-passage WJ-MSCs were seeded into 35-mm dishes (Corning) at a density of 20,000 cells/cm^2 in DMEM-F12 containing 10% FBS and 1% antibiotics (100 U/ml penicillin and 250 ng/ml streptomycin). On the second day, the medium was changed, and the cells were exposed to the medium with 20% FBS or treated for 24 h with the medium consisting of 20% FBS and 5-azacytidine (5-AZ; 3 μmol/L; Sigma-Aldrich). The medium was changed twice a week for 4 weeks or more until the

experiment was terminated. Control cells were cultured in DMEM-F12 supplemented with 5% FBS and antibiotics. CMG cells showed spontaneous beating after 5-AZ treatment for 1–2 weeks. 4′,6-Diamidino-2-phenylindole (DAPI; Sigma)-positive cardiomyogenic cells were stained with mouse monoclonal antibodies against sarcomeric α-actin, troponin T (TnT), connexin-43, and GATA-4 at 1:200 dilution (Beijing Biosynthesis Biotechnology Co., Ltd.) and FITC-conjugated secondary antibody (Beijing ZGB, Biotechnology Co., Ltd., Beijing, China; 1:100).

Microarray Experiment

RNA Extraction From WJ-MSCs and ESCs. Total RNA was extracted from three different samples each of third-passage WJ-MSCs and hESCs using Trizol reagent (Sigma-Aldrich). RNA was further purified using Qiagen RNeasy Mini Kit according to the manufacturer's instructions (Qiagen, Germantown, MD, USA). RNA quality was assessed by formaldehyde agarose gel (Sigma-Aldrich) electrophoresis (Bio-Rad, Hercules, CA, USA) and was quantitated spectrophotometrically (Thermo Scientific, Waltham, MA, USA).

Microarray Analysis. An aliquot of 2 μg of total RNA was used to synthesize double-stranded cDNA, then produce biotin-tagged cRNA using the MessageAmpTM II aRNA Amplification Kit (Takara, Shiga, Japan). The resulting bio-tagged cRNA were fragmented into strands of 35–200 bases in length according to the protocols from Affymetrix (Santa Clara, CA, USA). The fragmented cRNA was hybridized to a Human Genome U133 Plus 2.0 array (Affymetrix), containing 47,000 transcripts. Hybridization was performed at 45°C with rotation for 16 h (Affymetrix GeneChip Hybridization Oven 640). The GeneChip arrays were washed and then stained (streptavidin–phycoerythrin) on an Affymetrix Fluidics Station 450, followed by scanning on a GeneChip Scanner 3000 (Affymetrix). All samples were prepared in three biological repeats.

Normalization and Data Analysis. The hybridization data were analyzed using GeneChip Operating software (GCOS 1.4; Affymetrix). The scanned images were first assessed by visual inspection, then analyzed to generate raw data files saved as CEL files using the default setting of GCOS 1.4. An invariant set normalization procedure was performed to normalize the different arrays using a DNA-chip analyzer (dChip; Affymetrix). In a comparison analysis, 13,392 genes were determined to be significantly differentially expressed with a selection threshold of ratio >2.0 or ratio <0.5 in the output result.

Real-Time Quantitative Polymerase Chain Reaction Analysis

Selected genes, including embryonic stem cell marker genes and early cardiovascular-specific transcription factor genes from microarray experiments, were further confirmed by qRT-PCR (Table 1). Total RNA was extracted from three different samples each of WJ-MSCs and hESCs. Total RNA was reversed-transcribed using a QuantiTect Reverse Transcription kit (Applied Biosystems, Foster City, CA, USA). The qRT-PCR reactions were performed with Platinum SYBR Green qPCR superMix UDG (Invitrogen) using gene-specific primers (listed in Table 1; Sangon, Shanghai, China) following the manufacturer's instructions. PCR was performed in the Bio-Rad DNA

Table 1. Primers Used in Real-Time RT-PCR for Stemness Molecular Markers and Early Cardiac Transcription Factors Used in the Analysis of WJ-MSCs and hESCs

Gene	Sense Sequence	Antisense Sequence	Product Size (bp)
OCT4	ggggttctatttgggaaggtat	actcggttctcgatactggttc	209
SOX2	atgggttcggtggtcaagt	gctctggtagtgctgggacat	183
NANOG	aagaggtggcagaaaaacaact	tccctggtggtaggaagagtaa	102
PODXL	gataccccaaaacaccttctcc	gctgcttctcactctgtgtctg	93
SMAD3	ctgtgtgagttcgccttcaata	gaagttagtgttttcggggatg	177
Brachyury	acaaagagatgatggaggaacc	tccaaactgaggatgaggattt	119
KDR	ttcagagttggtggaacatttg	aacaggtgaggtaggcagagag	139
NKX2.5	gacatcctaaacctggaacagc	aaaggcagacgcacacttg	213
ISL1	tgatttccctatgtgttggttg	gttttcccatccctaacaaagc	157
KIT	cctggatgaaacgaatgagaat	acttctgggtctgtgagaggac	219
APJ	ccttcctctatgccttttcg	acaagggtctcctggctgtag	217
APLN	gctggaagacggcaatgt	cctccagagaagcagaccaat	214
THY1	aataccagcagttcacccatc	tgaaggcggataagtagaggac	160
GAPDH	agaaggctggggctcatttg	aggggccatccacagtcttc	258

WJ-MSCs, Wharton's jelly mesenchymal stem cells; hESCs, human embryonic stem cells; OCT4, octamer binding transcription factor-4; SOX2, sex determining region Y box 2; PODXL, podocalyxin-like; SMAD3, mothers against decapentaplegic homolog 3; KDR, kinase insert domain receptor; NKX2.5, NK2 homeobox 5; ISL1, islet-1; KIT, v-kit Hardy-Zuckerman 4 feline sarcoma viral oncogene homolog; APJ, apelin receptor; APLN, apelin; THY1, thymocyte cell surface antigen; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

Engine Opticon-2 (Bio-Rad, CA, USA) under the following conditions: 50°C for 2 min, 95°C for 10 min, 40 cycles (15 s at 95°C, 1 min at 60°C). Target genes were assayed in triplicate on each plate. Glyceraldehyde phosphate dehydrogenase (GAPDH) was used as a housekeeping gene to normalize, and then it was quantified in WJ-MSCs relative to the expression level in ESCs, after running dilution standards for every assay to ensure the ratio of the differences in cycle thresholds ($2^{-\Delta\Delta Ct}$) was equivalent to the fold change calculated from the standard curve.

Statistical Analysis

Data were presented as means ± standard error of the mean. Statistical comparisons were performed using either the Student's two-tailed *t* test (unpaired/paired) or the Wilcoxon matched pairs test as appropriate according to data distribution. Values of $p<0.05$ were considered significant. Data analysis and graphical representations were performed by using GraphPad Prism 5 software (GraphPad, San Diego, CA, USA). The differentially expressed genes were mapped to Gene Ontology (GO) terms and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways based on the human accession ID, using MAS (molecule annotation system, http://bioinfo.capitalbio.com/mas) platform. GO terms and KEGG pathways with false discovery rate (FDR)-corrected values of $p<0.05$ were considered statistically significant.

RESULTS

Morphology and Immunophenotypic Characterization

WJ-MSCs isolated from human Wharton's jelly with phase-contrast microscopy displays a uniform spindle-shaped morphology similar to fibroblastoid cells (Fig. 1A). In vitro differentiation analysis confirmed that WJ-MSCs exhibited the capacity to differentiate into various cell types, such as osteoblasts, chondrocytes, and adipocytes (Fig. 1B–D).

The population doubling time of WJ-MSCs at passages 5–6 was 30–36 h in this study. For further characterization of WJ-MSCs, a panel of surface markers was tested

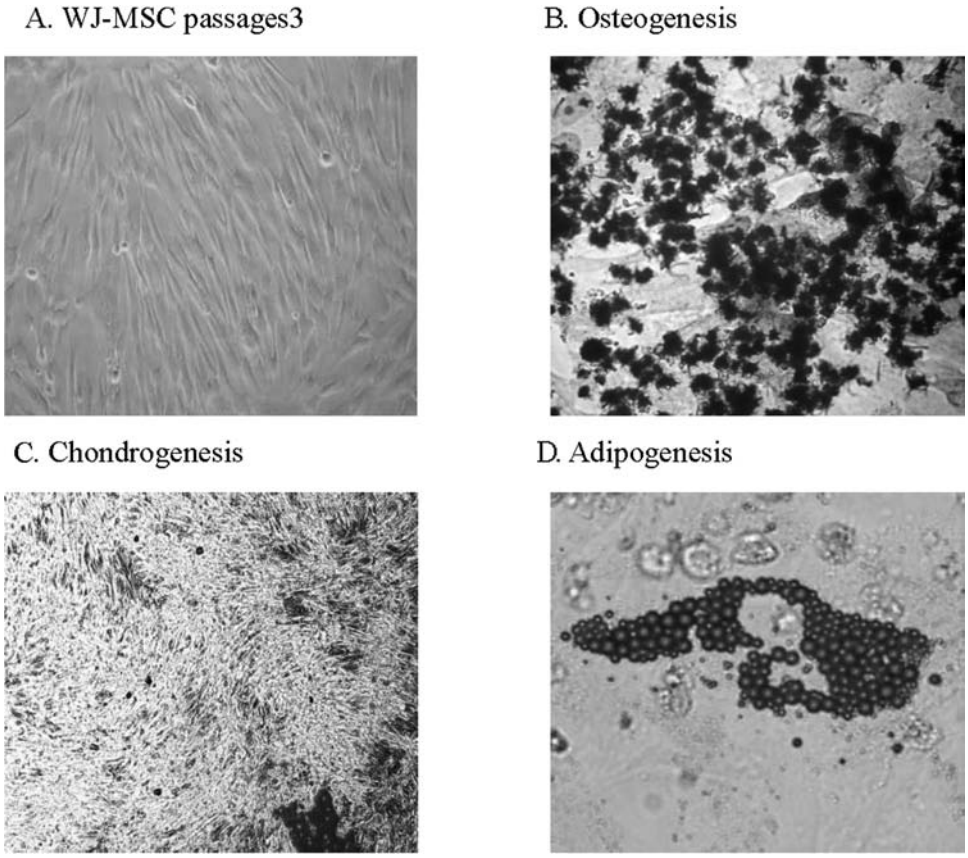

Figure 1. Morphology and multilineage differentiation capacity of WJ-MSCs. (A) Wharton jelly-derived mesenchymal stem cells (WJ-MSCs) showed a homogeneous spindle-shaped morphology. (B) Osteogenesis was analyzed by von Kossa staining for mineral nodule deposition. (C) Chondrogenesis was assessed by Safranin O staining for proteoglycan deposition. (D) Adipogenesis was observed by the presence of lipid vesicles and confirmed by oil red O staining.

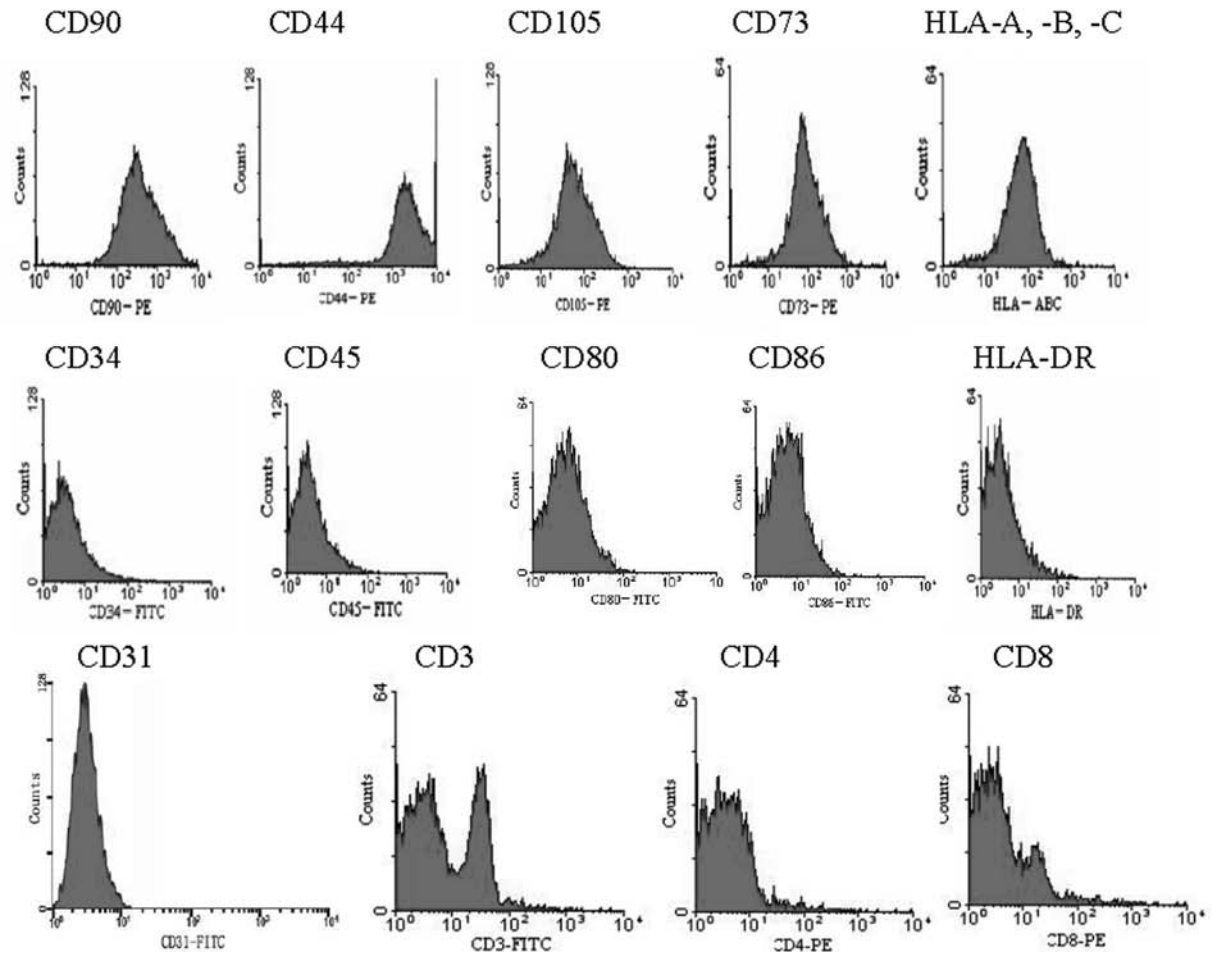

Figure 2. Immunophenotype of WJ-MSCs by flow cytometric analysis. Representative histograms are demonstrated. The WJ-MSCs were positive for cluster of differentiation 44 (CD44), CD73, CD90, CD105, and human leukocyte antigen (HLA)-A, -B, and -C and negative for CD31, CD34, CD45, CD3, CD4, CD8, CD80, CD86, and HLA-DR.

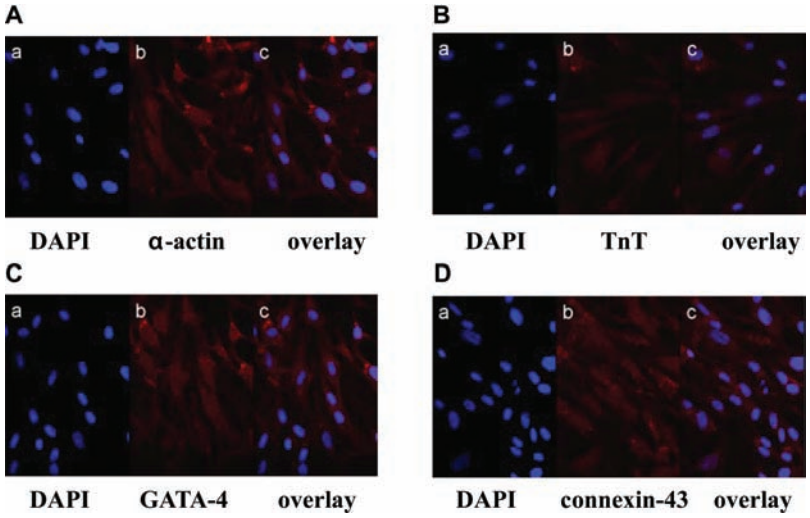

Figure 3. Confocal microscopy of fluorescent immunohistochemical staining of DAPI-labeled WJ-MSCs induced by 5-AZ after 2 weeks. Expression of (A) α-actin, (B) troponin T, (C) GATA-4, and (D) connexin-43. Nuclei identified by DAPI (a), all proteins—red fluorescence (b), merged image (c). 5-AZ, 5-azacytidine.

using flow cytometric analysis. WJ-MSCs were negative for CD31 (endothelial cell marker), CD34, CD45 (both as hematopoietic markers), HLA-DR (human leukocyte antigen class II), and CD3, CD4, CD8, CD80, and CD86, whereas they were positive for CD90, CD44 (both as adhesion markers), CD105, CD73 (both as mesenchymal markers), and HLA-A, -B, and -C (class I) (Fig. 2).

To confirm the in vitro capacity of WJ-MSCs to differentiate into cardiomyocytes, 2 weeks after 5-AZ treatment, we examined the expression of several cardiac myocyte markers. DAPI staining was used to identify WJ-MSCs (blue nucleus with multiple nucleoi). These cells stained positive for the cardiac specific proteins sarcomeric α-actin, TnT, the cardiac specific transcription factor GATA-4, and the intercellular connection and electrical coupling factor connexin-43 (Fig. 3).

Similarities and Differentiation of Gene Expression Profiles Between WJ-MSCs and hESCs

Using high-density oligonucleotide microarrays, we analyzed the global gene expression of WJ-MSCs and hESCs. Further comparison of gene expression in WJ-MSCs and hESCs indicated that 7,417 genes showed a twofold difference in expression. Of these, 2,951 were twofold or higher in WJ-MSCs than hESCs. The other 4,466 genes were downregulated in WJ-MSCs compared with ESCs. To generate the list of similar and differentially expressed genes, a fold-change ranking method was used to perform significance analysis of microarrays (54). After filtering with $p<0.05$, we ranked genes by no fold changes and chose the top 50 genes that were expressed similarly between WJ-MSCs and hESCs as shown in Figure 4. Molecular function, included major similar genes involved in ribosomal structural constituents (34 genes), protein binding (31 genes), RNA binding (19 genes), and oxidoreductase activity (7 genes). The top biological process associated with genes in this network, included those involvement in translational elongation, ribosomal RNA (rRNA) processing, ribosomal small subunit synthesis, mitochondrial electron transport, nicotinamide adenine dinucleotide (NADH)–ubiquinone interaction, ribosome biogenesis, assembly, and translational initiation. (Please contact the authors if you wish to view the functional network plots of this from the MetaCore program.)

After filtering with $p<0.05$, we ranked genes by twofold changes and chose the top 25 genes that were either upregulated or downregulated, respectively, in WJ-MSCs and hESCs as shown in Tables 2 and 3. Upregulated genes were involved in molecular functions, including protein binding, sequence-specific DNA binding, transcription factor activity, collagen binding, extracellular matrix structural constituent formation, platelet-derived growth factor binding, and ligand regulated transcription factor activity. The biological processes associated with genes included integrin-mediated signaling pathways,

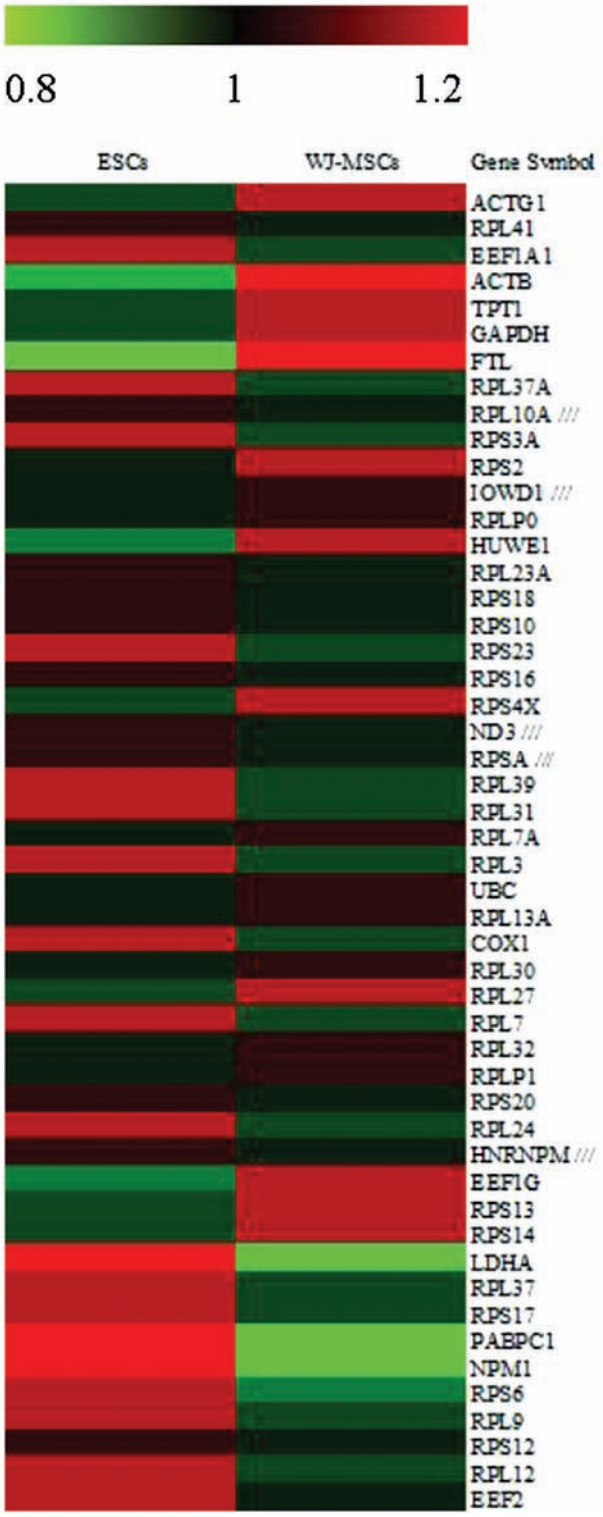

Figure 4. Heat map of top 50 of common genes expressed by human WJ-MSCs and ESCs. Log2 expression levels are shown in a green-black-red gradient. ESCs, embryonic stem cells.

Table 2. Top 25 Genes With Greater Than Twofold Upregulation in hWJ-MSCs Than hESCs

UniGene ID	Gene Title	Gene Symbol	WJ-MSCs	ESCs	Ratio
Hs.443625	collagen, type 3, α1	COL3A1	15,254.3	16.3	933.6
Hs.188401	annexin A10	ANXA10	2,980	4.49	663.7
Hs.1908	serglycin	SRGN	3,867.9	6.64	582.52
Hs.696554	integrin, β-like 1 (with EGF-like repeat domains)	ITGBL1	2,209.5	5.55	398.1
Hs.594481	serpin peptidase inhibitor, clade B (ovalbumin), member 2	SERPINB2	2,428.3	6.69	362.97
Hs.44276	homeobox C10	HOXC10	2,332.9	6.51	358.36
Hs.718429	decorin	DCN	11,368	31.85	356.92
Hs.459088	KIAA1199	KIAA1199	8,390.7	23.81	352.4
Hs.348522	potassium voltage-gated channel, Isk-related family, member 4	KCNE4	1,343.8	4.07	330.17
Hs.406475	lumican	LUM	6,884.7	24.28	283.55
Hs.503911	nicotinamide *N*-methyltransferase	NNMT	11,067	39.86	277.65
Hs.517033	transglutaminase 2 (C polypeptide, protein-glutamine-γ-glutamyltransferase)	TGM2	6,549.5	23.81	275.07
Hs.76152	aquaporin 1 (Colton blood group)	AQP1	889.16	3.77	235.85
Hs.386726	regulator of G-protein signaling 4	RGS4	6,330.4	27.63	229.11
Hs.233240	collagen, type 6, α3	COL6A3	9,260.3	40.65	227.81
Hs.567412	leucine-rich repeat containing 17	LRRC17	2,956.7	14.87	198.84
Hs.549040	homeobox C6	HOXC6	4,318.1	23.58	183.13
Hs.348365	caspase recruitment domain family, member 16	CARD16	300.87	1.65	182.35
Hs.40499	dickkopf homolog 1 (*Xenopus laevis*)	DKK1	11,955	66.01	181.11
Hs.436416	integrin, α11	ITGA11	1,475.4	8.79	167.85
Hs.519445	nuclear receptor subfamily 2, group F, member 1	NR2F1	3,088	21.18	145.8
Hs.715499	transmembrane 4 L six family member 1	TM4SF1	1,229.3	8.51	144.45
Hs.93764	carboxypeptidase A4	CPA4	6,089.2	44.63	136.44
Hs.65641	sterile α motif domain containing 9	SAMD9	134.99	1	134.99
Hs.153952	5′-nucleotidase, ecto (CD73)	NT5E	874.96	6.52	134.2

The signal intensity on the microarray is reported. Significantly differentially expressed with a selection threshold of ratio >2.0 or ratio <0.5 in the output result.

cell-matrix adhesion, anterior/posterior pattern formation, peptide cross-linking, collagen fibril organization, cell adhesion, development, and T-cell secretory granule organization. (Please contact the authors if you wish to view the functional network plots of this from the MetaCore program.)

Downregulated genes were involved in molecular functions, including zinc ion binding, transcription factor activity, metal ion binding, L-lysine transporter activity, and cationic amino acid transporter activity. The biological process associated with genes in the network included regulation of transcription, pre-B-cell differentiation, positive regulation of B-cell apoptosis, positive regulation of T-cell homeostatic proliferation, negative regulation of T-cell homeostatic proliferation, and B-cell receptor transport into membrane rafts. (Please contact the authors if you wish to view the functional network plots of this from the MetaCore program.)

Validation of Microarray Results With Real-Time Quantitative PCR

To verify and add to the array results, pivotal genes from microarray analysis that have been implicated in the stemness and early cardiovascular development were selected and subjected to quantitative RT-PCR. The genes selected were octamer binding transcription factor-4 (OCT4), sex determining region Y box 2 (SOX2), NANOG, podocalyxin-like (PODXL), mothers against decapentaplegic homolog 3 (SMAD3), Brachyury, kinase insert domain receptor (KDR), NK2 homeobox 5 (NKX2.5), islet-1 (ISL1), v-kit Hardy-Zuckerman 4 feline sarcoma viral oncogene homolog (KIT), apelin receptor (APJ), apelin (APLN), and thymocyte cell surface antigen (THY1). The mean (±SEM) relative expression (n=4 sample each) of each of these genes, as compared with the expression by quantitative PCR, is shown in Figure 5, which confirmed the good correlation between Affymetrix gene expression results and real-time quantitative PCR.

Core Gene Expression of Stemness in WJ-MSCs, Compared With hESC Controls

The International Stem Cell initiative (ISCI) characterized 59 human embryonic stem cell lines from 17 laboratories worldwide. Accordingly, we compared the expression difference of the stemness genes between WJ-MSC and hESC samples (Table 4). WJ-MSCs expressed most of the stemness marker genes, but most of these were expressed at lower levels than in hESCs. However, some key core genes such as thiosulfate sulfurtransferase [TST or stage-

Table 3. Top 25 Genes With Lower Than 2.5-Fold Downregulation in WJ-MSCs Than ESCs

UniGene ID	Gene Title	Gene Symbol	WJ-MSCs	ESCs	Ratio
Hs.86154	lin-28 homolog (*C. elegans*)	LIN28	11.38	11,720	0.001
Hs.653700	Zic family member 2 (odd-paired homolog, *Drosophila*)	ZIC2	5.15	5,089.7	0.001
Hs.2563	tachykinin, precursor 1	TAC1	3.38	3,111	0.0011
Hs.288655	orthodenticle homeobox 2	OTX2	5.39	4,880.7	0.0011
Hs.652553	hypothetical LOC790952	HESRG	15.54	12,904	0.0012
Hs.460789	TOX high mobility group box family member 3	TOX3	1	808.49	0.0012
Hs.489824	protein tyrosine phosphatase, receptor-type, Z polypeptide 1	PTPRZ1	3.68	2,954.4	0.0012
Hs.661360	Nanog homeobox	NANOG	13.15	8,812	0.0015
Hs.175220	solute carrier family 7 (cationic amino acid transporter, y^+ system), member 3	SLC7A3	11.69	7,241.5	0.0016
Hs.694721	CD24 molecule	CD24	26.99	16,382	0.0016
Hs.163244	leucine-rich repeat neuronal 1	LRRN1	5.21	2,966.6	0.0018
Hs.25960	v-myc myelocytomatosis viral related oncogene, neuroblastoma derived (avian)	MYCN	7.23	3,570.3	0.002
Hs.589848	par-6 partitioning defective 6 homolog β (*C. elegans*)	PARD6β	2.85	1,297.6	0.0022
Hs.135787	sal-like 1 (*Drosophila*)	SALL1	3.11	1,288.9	0.0024
Hs.1787	proteolipid protein 1	PLP1	4.22	1,675	0.0025
Hs.461086	cadherin 1, type 1, E-cadherin (epithelial)	CDH1	15.84	6,253.8	0.0025
Hs.685462	LINE-1 type transposase domain containing 1	L1TD1	19.12	7,159	0.0027
Hs.23616	lin-28 homolog B (*C. elegans*)	LIN28B	11.59	3,924.1	0.003
Hs.481022	secreted frizzled-related protein 2	SFRP2	7.12	2,299.1	0.0031
Hs.254097	PHD finger protein 21B	PHF21B	1.96	616.99	0.0032
Hs.487471	epithelial splicing regulatory protein 1	ESRP1	12.52	3,898.3	0.0032
Hs.542050	epithelial cell adhesion molecule	EPCAM	23.04	7,172.2	0.0032
Hs.690098	podocalyxin-like	PODXL	26.03	7,801.7	0.0033
Hs.665450	major histocompatibility complex, class II, DP β2 (pseudogene)	HLA-DPβ2	5.76	1,665.1	0.0035
Hs.385870	teratocarcinoma-derived growth factor 1 /// teratocarcinoma-derived growth factor 3, pseudogene	TDGF1 /// TDGF3	33.4	9,627.1	0.0035

The signal intensity on the microarray is reported. Significantly differentially expressed with a selection threshold of ratio >2.0 or ratio <0.5 in the output result.

specific embryonic antigen 3 (SSEA3)], transducing-like enhancer of split 1 [TLE1 or enhancer of split groucho-like protein 1 (ESG1)], HLA-E, HLA-B, HLA-C, HLA-G, and HLA-A were higher than in hESCs (Table 4).

Expression of Early Cardiac Transcription Factor Genes in WJ-MSCs, Compared With hESC Controls

As shown in Table 5, most of the early cardiovascular progenitor genes were expressed in WJ-MSCs. Moreover, some key cardiac transcription factors, such as T box 3 (TBX3), TBX2, TBX20, chemokine (C-X-C motif) receptor 5 (CXCR5), NKX2-5, MEF2C (myocyte enhancer factor 2C), TBX1, KITLG (stem cell factor; SCF), CXCR3, TBX4, CXCR6, TBX5, TBX6, TBX19, TBX10, and PECAM1 (platelet/endothelial cell adhesion molecule 1; CD31), were expressed at higher levels than in hESCs.

Functional Analysis of Signature Gene Expression in WJ-MSCs, Compared With hESC Controls

To generate the list of differentially expressed signature genes, a fold-change ranking method was done as described above. Similarities in pathways affected ribosomes, adherens junctions, focal adhesion, and tight junctions in WJ-MSCs and hESCs. However, upregulation of pathways was involved in extracellular matrix (ECM)–receptor interaction, nicotinate and nicotinamide metabolism, focal adhesion, TGF-β signaling pathway, and pyrimidine metabolism in WJ-MSCs compared with hESCs. Downregulation in the pathway was involved in endometrial cancer, hedgehog signaling pathway, adherens junction, cell adhesion molecules (CAMs), tight junction, and Wingless-type mouse mammary tumor virus (MMTV) integration site family (Wnt) signaling pathway in WJ-MSCs compared with

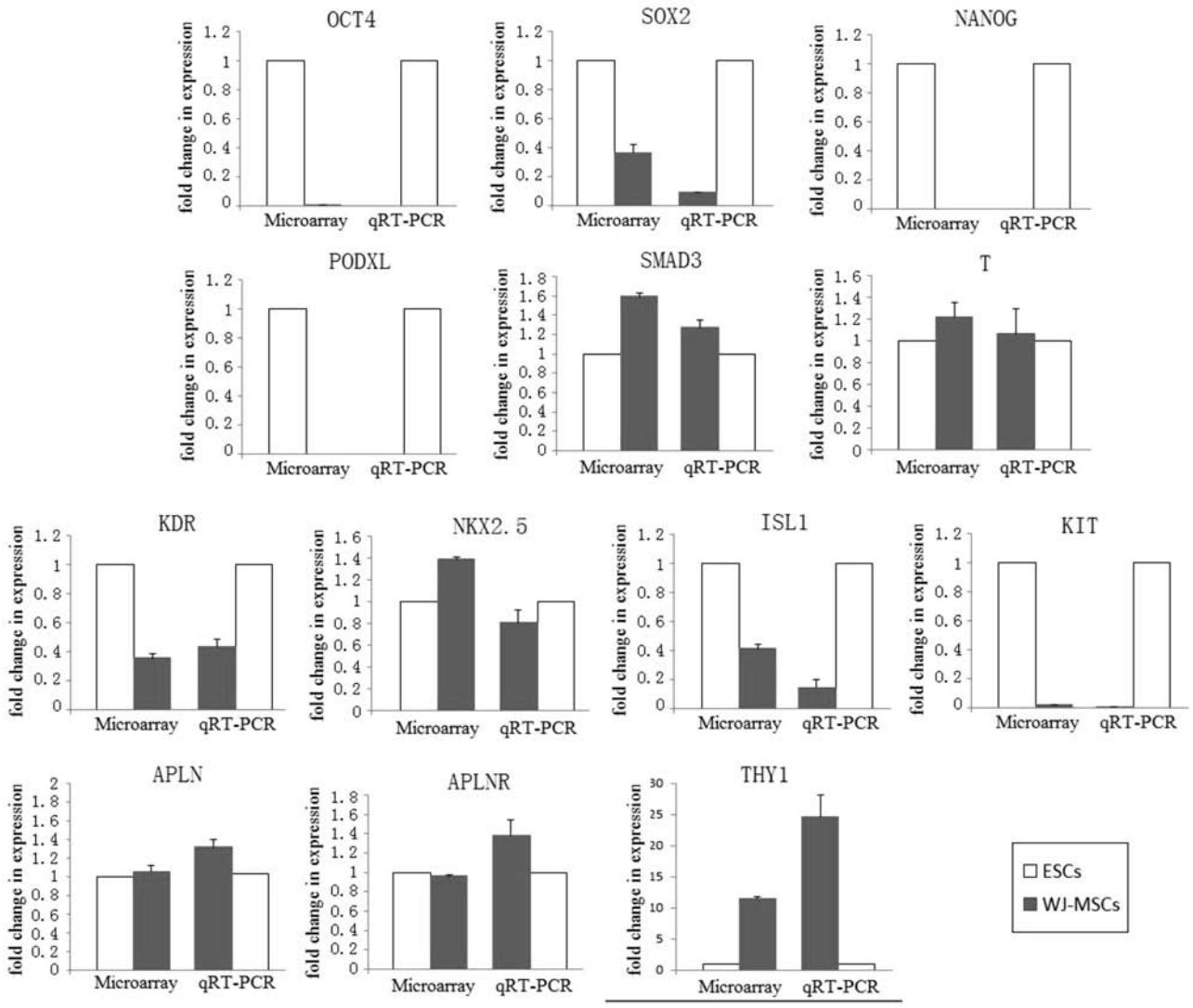

Figure 5. Comparison of gene expression by microarray and qRT-PCR. Comparisons of octamer binding transcription factor-4 (OCT4), sex determining region Y box 2 (SOX2), NANOG, podocalyxin-like (PODXL), mothers against decapentaplegic homolog 3 (SMAD3), Brachyury, kinase insert domain receptor (KDR), NK2 homeobox 5 (NKX2.5), islet-1 (ISL1), v-kit Hardy-Zuckerman 4 feline sarcoma viral oncogene homolog (KIT), apelin receptor (APJ), apelin (APLN), and thymocyte cell surface antigen (THY1). Gene expression patterns as determined by microarray and qRT-PCR analysis. The *x*-axis indicates the WJ-MSCs and control hESCs, and the *y*-axis represents fold changes determined by microarray and qRT-PCR analysis.

hESCs. (Please contact the authors if you wish to view the functional network plots of this from the MetaCore program.)

Functional Analysis of Immune Property Gene Expression in WJ-MSCs, Compared With hESC Controls

In this study, we demonstrated greater expression of genes that regulate immune properties in WJ-MSCs than in hESCs, as shown in Table 6, such as: 5′-nucleotidase, ecto (CD73), endoglin, Thy-1 cell surface antigen (Thy-1), CD44 molecule, membrane metallo-endopeptidase (MME), CD14 molecule, major histocompatibility complex class I, G (HLA-G), selectin P, and integrin (granule membrane protein antigen CD62). These data indicate that a proper immune characterization of WJ-MSC lines should be based on a more extended panel of marker genes, spanning HLA typing to costimulatory molecule expression.

Functional Analysis of Growth Factors and Receptor Gene Expression in WJ-MSCs, Compared With hESC Controls

WJ-MSCs have been shown to express a broad spectrum of cytokines and growth factor genes as shown in

Table 4. Core Gene Expression of Stemness in WJ-MSCs, Compared With hESCs

UniGene ID	Gene Title	Gene Symbol	WJ-MSCs	ESCs	Ratio
Hs.143507	T, brachyury homolog (mouse)	T	151.28	124.51	1.215
Hs.661360	Nanog homeobox	NANOG	13.15	8,812	0.0015
Hs.450254	POU class 5 homeobox 1 ///	POU5F1 (OCT4) ///			
	POU class 5 homeobox 1B /// POU class 5 homeobox 1 pseudogene 3 /// POU class 5 homeobox 1 pseudogene 4	POU5F1B /// POU5F1P3 /// POU5F1P4	42.04	7,798.9	0.0054
Hs.518438	SRY (sex determining region Y)-box 2	SOX2	33.96	109.08	0.3113
Hs.86154	lin-28 homolog (*C. elegans*)	LIN28	11.38	11,720.3	0.00097
Hs.690098	podocalyxin-like	PODXL (GCTM2)	26.03	7,801.7	0.0033
Hs.390420	fucosyltransferase 4 [α-(1,3)-fucosyltransferase, myeloid-specific]	FUT4 (SSEA1)	344.23	193.73	1.77685
Hs.474783	thiosulfate sulfurtransferase (rhodanese)	TST (SSEA3)	2,076.3	357.57	5.8066
Hs.618504	SMAD family member 3	SMAD3 (SSEA4)	69.16	44.3	1.5612
Hs.376206	Kruppel-like factor 4 (gut)	KLF4	54.66	52.33	1.0445
—	v-myc myelocytomatosis viral oncogene homolog (avian)	MYC	1,429.9	6,023.8	0.2374
Hs.385870	teratocarcinoma-derived growth factor 1 ///	TDGF1 (CRITPO) ///	33.4	9,627.1	0.0035
	teratocarcinoma-derived growth factor 3, pseudogene	TDGF3			
Hs.335787	zinc finger protein 42 homolog (mouse)	ZFP42 (REX1)	15.63	515.47	0.0303
Hs.197320	transducin-like enhancer of split 1 [E(sp1) homolog, *Drosophila*]	TLE1 (ESG1)	254.68	126.12	2.0193
Hs.351113	developmental pluripotency associated 2	DPPA2	36.59	521.84	0.07012
Hs.317659	developmental pluripotency associated 4	DPPA4	43.44	47.68	0.9111
Hs.492203	telomerase reverse transcriptase	TERT	39.78	180.73	0.2201
Hs.719101	Thy-1 cell surface antigen	THY1 (CD90)	7,908.9	696.3	11.359
Hs.650174	major histocompatibility complex, class I, E	HLA-E	5,043.6	948.54	5.3172
Hs.703277	major histocompatibility complex, class I, B	HLA-B	3,396.2	669.23	5.0748
Hs.654404	major histocompatibility complex, class I, C	HLA-C	4,668.5	1,386.7	3.3667
Hs.512152	major histocompatibility complex, class I, G	HLA-G	1,031.1	376.79	2.7366
Hs.181244	major histocompatibility complex, class I, A	HLA-A	4,162.8	1,582.7	2.6302
Hs.114286	CD9 molecule	CD9	2,901.3	2,201.9	1.3177
Hs.519972	major histocompatibility complex, class I, F	HLA-F	52.62	50.03	1.0518
Hs.86232	growth differentiation factor 3	GDF3	160.61	259.25	0.6195
Hs.302352	γ-aminobutyric acid (GABA) A receptor, β3	GABRβ3	12.6	547.77	0.023

The signal intensity on the microarray is reported. Significantly differentially expressed with a selection threshold of ratio >2.0 or ratio <0.5 in the output result.

Figure 6. After ranking genes by twofold or more changes, we found that ACTA2 (actin, α2, smooth muscle, aorta), TGFI, IL-6 (interleukin 6), VEGFC (vascular endothelial growth factor C), IL-1B, IL-7, TGF-β2, HRAS (v-Ha-ras Harvey rat sarcoma viral oncogene homolog), NGF (nerve growth factor), HGF (hepatocyte growth factor), BMP6 (bone morphogenetic protein 6), MMP1 (matrix metallopeptidase 1), IL-8, PDGFC (platelet-derived growth factor C), HIF1A (hypoxia inducible factor 1, α subunit), BMP4, IFNβ1 (interferon, β1), FGF7 (fibroblast growth factor 7), (keratinocyte growth factor) KGF (IL)15, and THOP1 (thimet oligopeptidase 1) exhibited much higher expression in WJ-MSCs than in hESCs.

DISCUSSION

WJ-MSCs originate from embryonic epiblasts and possess properties between hESCs and adult stem cells, and are thought to be more primitive than MSCs from any other adult tissue sources (8,45,53,56). They represent an important and rich source of primitive cells and are now considered as an alternative source of stem cells (8,45,53,56). However, the properties of stemness

Table 5. Expression of Early Cardiac Transcription Factor Genes in WJ-MSCs, Compared With hESCs

UniGene ID	Gene Title	Gene Symbol	WJ-MSCs	ESCs	Ratio
Hs.714737	T-box 3	TBX3	1,077.06	157.06	6.85763
Hs.531085	T-box 2	TBX2	566.13	178.33	3.17462
Hs.404167	T-box 20	TBX20	38.99	15.53	2.51062
Hs.113916	chemokine (C-X-C motif) receptor 5	CXCR5	77.29	53.37	1.44819
Hs.54473	NK2 transcription factor related, locus 5 (*Drosophila*)	NKX2-5	357.88	254.47	1.40637
Hs.653394	myocyte enhancer factor 2C	MEF2C	64.86	46.57	1.39274
Hs.173984	T-box 1	TBX1	44.36	32.51	1.3645
Hs.1048	KIT ligand	KITLG (stem cell factor)	38.84	29.22	1.32923
Hs.272409	T-box 21	TBX21	63.26	48.6	1.30165
Hs.198252	chemokine (C-X-C motif) receptor 3	CXCR3	73.83	59.63	1.23814
Hs.143907	T-box 4	TBX4	77.96	64.27	1.21301
Hs.34526	chemokine (C-X-C motif) receptor 6	CXCR6	75.87	65.04	1.16651
Hs.381715	T-box 5	TBX5	54.06	47.08	1.14826
Hs.198301	T-box 6	TBX6	100.41	88.32	1.13689
Hs.716656	T-box 19	TBX19	84.59	76.21	1.10996
Hs.454480	T-box 10	TBX10	59.06	56.38	1.04753
Hs.514412	platelet/endothelial cell adhesion molecule	PECAM1 (CD31)	69.84	68.66	1.01719
Hs.251830	T-box 18	TBX18	100.54	102.24	0.98337
Hs.146196	T-box 15	TBX15	44.79	46.18	0.9699
Hs.243987	GATA binding protein 4	GATA4	33.38	70.99	0.47021
Hs.374253	T-box 22	TBX22	5.91	13.13	0.45011
Hs.505	ISL LIM homeobox 1	ISL1	38.45	86.1	0.44657
Hs.479756	kinase insert domain receptor (a type 3 receptor tyrosine kinase)	KDR	134.18	417.56	0.32134
Hs.471751	chemokine (C-X-C motif) receptor 7	CXCR7	9.34	37.03	0.25223
Hs.479754	v-kit Hardy-Zuckerman 4 feline sarcoma viral oncogene homolog	KIT	29.5	1,478.02	0.01996
Hs.593413	chemokine (C-X-C motif) receptor 4	CXCR4	12.64	2,199.63	0.00575

The signal intensity on the microarray is reported. Significantly differentially expressed with a selection threshold of ratio >2.0 or ratio <0.5 in the output result.

molecules in WJ-MSCs, compared with that in hESCs, have not previously been clarified. Furthermore, both similarities and differences in global gene profiles between WJ-MSCs to hESCs were not previously established. In the present study, we isolated WJ-MSCs by a nonenzymatic method. WJ-MSCs express a set of core mesenchymal stem cell markers and are able to differentiate into osteoblasts, chondrocytes, and adipocytes. Further, using microarray analysis by Affymetrix GeneChip and functional network analyses, we determined the degree of expression of stemness genes exhibited by the Human Stem Cell Pluripotency array, containing markers of characterized undifferentiated stem cells. We also defined a wide range of stem cell gene expression in the WJ-MSCs and compared the degree of expression with hESCs. At same time, the definitive markers of early cardiac precursor cells and more committed progenitors were further characterized in WJ-MSCs.

Our results demonstrated for the first time that WJ-MSCs had significant expression of the undifferentiated human embryonic stem cell core markers, such as SOX2, NANOG, LIN28, SSEA1, SSEA3, SSEA4, KLF4, c-MYC, TDGF1, and ZFP42, with a relatively lower level of expression than in hESCs. It is also a surprise that we found that WJ-MSCs have high expression of early cardiac transcription factors, such as KDR, islet 1 (Isl-1), and Nkx2.5, some of which exhibit higher levels of expression in WJ-MSCs than in hESCs. Functional analysis revealed the signature gene of WJ-MSCs with specific roles involved in immune regulation, cytoskeletal regulation, chemokine regulation, cell adhesion, and cell signaling.

The key characteristics of stem cells are long-term self-renewal and a capacity to differentiate into diverse mature tissues, which favor their use in regenerative medicine. Human MSCs have been isolated from bone marrow,

Table 6. Expression of Immune Property Genes in WJ-MSCs, Compared With hESCs

UniGene ID	Gene Title	Gene Symbol	WJ-MSCs	ESCs	Ratio
Hs.153952	5′-nucleotidase, ecto (CD73)	NT5E	874.96	6.52	134.2
Hs.76753	endoglin	ENG (CD105)	2,050.62	67.45	30.4021
Hs.719101	Thy-1 cell surface antigen	THY1	7,908.9	696.3	11.359
Hs.502328	CD44 molecule (Indian blood group)	CD44	790.59	61.89	12.7741
Hs.307734	membrane metallo-endopeptidase	MME (CD10)	2,836.2	339.21	8.36119
Hs.163867	CD14 molecule	CD14	109.97	40.14	2.73966
Hs.512152	major histocompatibility complex, class I, G	HLA-G	1,031.1	376.79	2.7366
Hs.73800	selectin P (granule membrane protein 140 kDa, antigen CD62)	SELP	72.35	45.65	1.58488
Hs.482077	integrin, α2 (CD49B, α2 subunit of VLA-2 receptor)	ITGA2	278.87	203.41	1.37097
Hs.436873	integrin, αV (vitronectin receptor, α polypeptide, antigen CD51)	ITGAV	3,546.13	2,623.41	1.35173
Hs.374990	CD34 molecule	CD34	178.29	137.93	1.29261
Hs.85258	CD8a molecule	CD8A	50.34	40.46	1.24419
Hs.712553	membrane-spanning 4-domains, subfamily A, member 1	MS4A1 (CD20)	12.86	11.49	1.11923
Hs.631659	CD4 molecule	CD4	59.7	56.96	1.0481
Hs.83731	CD33 molecule	CD33	46.69	51.16	0.91263
Hs.643813	integrin, β1 (fibronectin receptor, β polypeptide, antigen CD29 includes MDF2, MSK12)	ITGB1 (CD29)	18.5	21.39	0.86489
Hs.654514	protein tyrosine phosphatase, receptor type, C	PTPRC (CD45)	30.46	43.16	0.70575
Hs.218040	integrin, β3 (platelet glycoprotein IIIa, antigen CD61)	ITGB3	43.52	111.93	0.38881
Hs.593413	chemokine (C-X-C motif) receptor 4	CXCR4 (CD184)	12.64	2,199.63	0.00575
Hs.614734	prominin 1	PROM1	6.35	1,302.33	0.00488
Hs.694721	CD24 molecule	CD24	26.99	16 381.8	0.00165

The signal intensity on the microarray is reported. Significantly differentially expressed with a selection threshold of ratio >2.0 or ratio <0.5 in the output result.

adipose tissues, cord blood, amniotic fluid, amniotic membrane, placenta, umbilical cord tissues, and Wharton's Jelly of the umbilical cord (18,28,40,49,54,57,64). Tsai et al., using microarray analysis of MSCs derived from four different origins including amniotic fluid, amniotic membrane, cord blood, and bone marrow, found that within each group of MSCs from the same origin, the variability of the gene expression levels was smaller than that between groups of different origins, and functional genomic studies revealed the specific roles of MSCs from different origins (48,54). However, so far there have been no reports on the global profile of genes in WJ-MSCs and their functional analysis, and no comparison with hESCs. In the present study, we used high-density oligonucleotide microarrays, Human Genome U133 Plus 2.0 array containing 47,000 transcripts, and functional network analyses. The Affymetrix microarray data were analyzed with GeneSifter software. There were 7,417 genes that showed a twofold or greater difference in expression between the WJ-MSCs and hESCs. Of these, 2,951 were twofold or higher in WJ-MSCs than in ESCs. The remaining 4,466 genes were downregulated in WJ-MSCs compared with ESCs. After filtering with a threshold of $p<0.05$, we ranked genes by the number of fold changes and chose the top 50 genes with similar expression between WJ-MSCs and hESCs. These genes are involved in molecular functions, such as coding for structural ribosome constituents, protein binding, RNA binding, oxidoreductase activity, and nucleotide binding.

The ISCI was established by the International Stem Cell Forum to carry out a comparative study of a large and diverse set of hES cell lines derived and maintained in different laboratories worldwide. The present ISCI study analyzed 59 independently derived hES cell lines from 17 laboratories in 11 countries. All lines exhibited similar expression patterns for several markers of human embryonic stem cells (51). In our study, WJ-MSCs were isolated from Wharton's jelly in an indistinct region of umbilical cord, which is a continuum from the subamnion to the perivascular region. In contrast to the gene markers established by ISCI, we found that WJ-MSCs expressed the majority of stemness core markers, such as NANOG,

Figure 6. Heat map of the expression of growth factors and receptor gene in WJ-MSCs, compared with hESCs. Log2 expression levels are shown in a green–black–red gradient.

SOX2, and LIN28, which notably maintain the undifferentiated ES cell state, although the levels were lower than the signal intensity reported on the microarray. Moreover, the group of genes whose expression correlated closely with that of NANOG and appear to be characteristic of the hES cell, such as MYC, TDGF1, growth differentiation factor 3 (GDF3), γ-aminobutyric acid (GABA) A receptor, β3 (GABRB3), and THY1, are also expressed by WJ-MSCs. To our surprise, we found that SSEA1, SSEA3, and SSEA4, which were another common set of markers generally to monitor the presence of pluripotent stem cells set up by ISCI, were highly expressed in WJ-MSCs, in comparison to the hESCs. The findings agree with recent reports in which it was found that MSCs derived from porcine or human Wharton's jelly of umbilical cord expressed some embryonic stem cells marker genes such as OCT-4, GATA4, KIT, Laminin, γ1 (LAMC1), LIN28, Noggin, nuclear receptor subfamily 6, group A, member 1 (NR6A1), POU5F1, runt-related transcription factor 2 (RUNX2), and SOX17 (10,15). Therefore, these data have demonstrated that WJ-MSCs have properties of primitive pluripotent stem cells and have undergone an unprecedented characterization process in our studies that further extend the similarities with respect to hESCs. The fact that WJ-MSCs remain behind and coexist with the hESCs suggests that it is possible that, during development, cells in the Wharton's jelly migrate into the fetus along with the primordial germ cells and hematopoietic cells to the aorta-gonad mesonephros (AGM) region (8,53). Moreover, in our study, WJ-MSCs express CXCR3, CXCR4, and CXCR6, which are the chemokine and surface receptors for function of the migration and homing ability. Thus, the WJ-MSCs express some genes characteristic of primitive stem cells including embryonic stem cells.

The most important finding in this study was that prominent functional genes that may be involved in cardiovascular formation and regeneration are expressed in WJ-MSCs. We found higher expression of mesoderm and specialized cardiac progenitor cell genes, including Brachyury T, mesoderm posterior 1 (Mesp1), KDR, Nkx2.5, and Isl-1. We also demonstrated that WJ-MSCs express GATA-4 and connexin-43, which are typically expressed in embryonic and myocardial cells. Indeed, our in vitro study showed that after 2 weeks of treatment with the DNA demethylation agent 5-azacytine, the WJ-MSCs differentiated into cardiomyocyte-like cells as shown in Figure 3. Immunostaining showed that differentiated cells were strongly positive for cardiac α-actin, TnT, connxin43, and GATA-4.

At present, some studies have demonstrated that the formation of the mature multichambered heart requires the contribution of diverse cell types with specialized function, including cardiomyocytes, endothelial cells, and vascular smooth muscle cells (20,50,61). Precursors for heart-forming cells in the vertebrate mesoderm transition from expressing Brachyury T to expressing mesoderm posterior 1 (Mesp1) when they enter the precardiac mesoderm stage of development (61). During Mesp1 cell migration, cardiac progenitor cells expand rapidly to form the anterior and lateral plate mesoderm, where they generate a crescent-shaped structure called the cardiac crescent (61). In the cardiac crescent stage, the heart precursor cells commit to the cardiac lineage and become cardiac progenitor cells expressing key developmental transcription factors such as KDR, Nkx2.5, and Isl-1 (7,20,50,61). Studies have identified that a diverse set of human fetal ISL-1$^+$ cardiovascular progenitors can give rise to the cardiomyocyte, smooth muscle and endothelial cell lineages (7,20,50). KDR is a cardiovascular progenitor that represents one of the earliest stages in mesoderm specification to the cardiovascular lineage (20,61). Yang et al. found that KDR$^+$ embryonic stem cell-derived population can differentiate into cardiac, endothelial, and vascular smooth muscle. When plated in monolayer cultures, KDR$^+$ cells differentiate to generate populations consisting of greater than 50% contracting cardiomyocytes (62). Nkx2.5 is a key transcription factor in cardiac development. It has been shown that cells in the first heart field, marked by Nkx2.5, give rise to the left ventricle and portions of the right and left atrium (7,20,50,61). Moretti's study found the ISL1$^+$/NKX2.5$^+$/KDR$^+$ mesodermal progenitors can effectively induce cardiovascular cell types (34). Therefore, a subpopulation of WJ-MSCs, as KDR$^+$/Isl-1$^+$/Nkx2.5$^+$ WJ-MSCs, may hold tremendous promise for cardiovascular regeneration medicine.

Recent studies found that the therapeutic capacity of MSCs to treat a wide spectrum of diseases has been attributed to their potential to differentiate into many different reparative cell types (33,42,52); however, the efficiency of transplanted MSCs to differentiate into functional reparative cells in the injured tissues or organs and the therapeutically relevant numbers have never been adequately documented or demonstrated (13,16). Recent reports have suggested that some of these reparative effects are not mediated by differentiation of MSCs but rather by paracrine factors secreted by MSCs (9,23). At present, our study found that WJ-MSCs express huge number of cytokines, chemokines, and growth factors at the transcript level by using a high-throughput genome-wide gene expression assay. The predominance of cytokines and chemokines such as TGF-β, VEGF, HGF, BMP, PDGF, FGF, MMP, and interleukins (IL-1–IL-17), which are involved in important signaling pathways in cardiovascular biology, bone development, and hematopoiesis, the majority of which have already been implicated in many aspects of cardiovascular, musculoskeletal, and hematopoiesis biology. For example, TGF-β is critical for correct heart development cardiac

remodeling, progression to heart failure, and vascularization (5,14). Janus kinase 3–signal transducer and activator of transcription 3 (JAK-STAT) signaling is associated with cardioprotection (36). Therefore, our present study also supports the notion that WJ-MSCs mediate paracrine effects for cardiac repair and regeneration.

On the other hand, we also found that WJ-MSCs exhibit properties of gene profiles unique to embryonic stem cells. Thus, these cells expressed a set of molecules, such as HLA-G and class 1 HLA molecules, but not class 2 HLA molecules, which support their ability to potentially induce immune tolerance (58). Especially in WJ-MSCs, the expression of HLA-G, which is one the main molecules responsible for induction of tolerance of natural killer (NK) cells toward self-cells, as well as in the process of tolerance of the mother's immune system toward the semiallogeneic embryo, is a key feature of the allogenesis stem cell transplantation process (35).

CONCLUSION

WJ-MSCs, as the dominant cells of extraembryonic derived tissue, share several features of gene expression compared to embryonic stem cells. They express in common a set of core stemness gene markers with hESCs, albeit in low levels. They also share the expression of early cardiac transcription factors, at levels even higher than hESCs. Like hESCs, they express CXCR3 and CXCR4 and so are likely to possess migratory and homing ability. Moreover, WJ-MSCs express huge numbers of cyokines, growth factors, and chemokines similar to hESCs. However, WJ-MSCs exhibit a gene profile unique to embryonic stem cells, in that they express HLA-G and class 1 HLA molecules and lack class 2 HLA molecules. It is therefore important to consider the benefits of WJ-MSCs, which are not ethically sensitive, have differentiation potential, and do not have the worrying issue of teratoma formation. Thus, WJ-MSCs constitute a true stem cell population and are promising cells for stem cell-based therapies.

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