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Human Wharton's Jelly Stem Cells Have Unique Transcriptome Profiles Compared to Human Embryonic Stem Cells and Other Mesenchymal Stem Cells

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Abstract The human umbilical cord that originates from the embryo is an extra-embryonic membrane and the Wharton's jelly within it is a rich source of stem cells (hWJSCs). It is not definitely known whether these cells behave as human embryonic stem cells (hESCs), human mesenchymal stem cells (hMSC) or both. They have the unique properties of high proliferation rates, wide multipotency, hypoimmunogenicity, do not induce teratomas and have anticancer properties. These advantages are important considerations for their use in cell based therapies and treatment of cancers. In a search for properties that confer these advantages we compared a detailed transcriptome profiling of hWJSCs using DNA microarrays with that of a panel of known hESCs, hMSCs and stromal cells. hWJSCs expressed low levels of the pluripotent embryonic stem cell markers including POUF1, NANOG, SOX2 and LIN28, thus explaining why they do not produce teratomas. Several cytokines were significantly upregulated in hWJSCs including IL12A which is associated with the induction of apoptosis, thus explaining their anticancer properties. When GO Biological Process analysis was compared between the various stem cell types, hWJSCs showed an increased expression of genes associated with the immune system, chemotaxis and cell death. The ability to modulate immune responses makes hWJSCs an important compatible stem cell source for transplantation therapy in allogeneic settings without immunorejection. The data in the present study which is the first detailed report on hWJSC transcriptomes provide a foundation for future functional studies where the exact mechanisms of these unique properties of hWJSCs can be confirmed.

Keywords DNA microarray · Human Wharton's jelly stem cells · Stem cells · Transcriptome

Chui-Yee Fong and Li-Ling Chak equally contributed to this work.

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Introduction

Stem cells in the human can be broadly classified as hematopoietic (hHSC), mesenchymal (hMSC) and embryonic (hESC) based on their growth behaviour, characterization, plasticity and other properties. Embryonic stem cells were observed for the first time in human preimplantation embryos in 1994 [1] and later cell lines were derived [2, 3]. Today, a variety of desirable tissues have been successfully derived from hESCs and some of these tissues have been transplanted with successful functional outcome in animal disease models [4–6]. However there have been concerns of immunorejection after transplantation since

hESC-derived tissue transplantation is in an allogeneic setting. Attempts are being made to circumvent this problem by personalization of tissues through somatic cell nuclear transfer (NT) and human induced pluripotent stem cell (hiPSC) technology [7–9]. Unfortunately, because of their pluripotency the tissues derived from hESCs, NT stem cells (NTSCs) and hiPSCs produce teratomas in laboratory animals [10]. There has been concern that teratomas may be induced in humans after transplantation of hESC, hiPSC or NTSC-derived tissues presumably induced by rogue undifferentiated hESCs, hiPSC or NTSCs residing in the derived tissues and this is currently a hurdle that has to be overcome before such tissues are taken to the clinic [11, 12].

On the other hand, MSCs are less problematic, can self-renew and are multipotent [13, 14]. The MSC of choice that is used in most research and clinical applications are those harvested from the human bone marrow (hBMMSC) [15]. However, such MSCs require painful invasive harvest; numbers are limited and their stemness properties do not last for too long *in vitro*. During our search for a stem cell that may not have such drawbacks, we studied the stem cells derived from the Wharton's jelly of human umbilical cords (hWJSCs) [16]. Although hWJSCs originate from the epiblast of the human embryo [14, 17], they are non-controversial as umbilical cords are discarded at birth. They can be harvested in abundance, are multipotent and they do not produce teratomas when injected into immunodeficient SCID mice [16]. Moreover, they have proliferative growth and retain their stemness properties for a long time *in vitro* (9–10 passages). Biochemical and immunohistochemical studies show that they have some MSC markers that are highly expressed (CD105, CD90, CD44), while cell-surface markers for ESCs (Tra-1-60, Tra-1-81, and SSEA-4) are expressed at low levels [16]. hWJSCs and human umbilical cord perivascular cells (HUCPVCs) have also been recently derived and characterized by other workers [18–21]. Troyer and Weiss [22] studied in detail the immune properties of hWJSCs using immunology protocols and concluded that there was no evidence for frank immunorejection of undifferentiated hWJSCs *in vivo* and concluded that they would be tolerated well in allogeneic transplantation settings. Thus far, hWJSCs have been differentiated into pancreatic islets and their engraftment confirmed in diabetic mice [19], into dopaminergic neurons with successful functional outcome in Parkinsonian rats [23] and recently they have been successfully differentiated into cartilage [20] and bone [24].

Ganta et al. [25] reported that rat WJSCs administered intravenously or intratumorally into rats carrying mammary adenocarcinomas abolished the tumours from day 14 onwards compared to sham-transplanted rats suggesting that rat WJSCs had anticancer properties. The regression of the tumours was completed by days 34 to 38 with no evidence of metastasis or recurrence at 100 days post-tumour inoculation. The mechanism of action of the anticancer effect of rat WJSCs was not clearly defined although the authors suggested that it may be via cell to cell contact or cell contact independent. Ayuzawa et al. [26] extended this finding to hWJSCs when they reported that naïve hWJSCs significantly attenuated the growth of human breast cancer cells in mice. In pilot studies we observed that hWJSCs at initial seeding doses of 100,000 cells had an apoptotic effect on equal numbers of breast cancer (MCF-7), colorectal (HT-29) and hepatocarcinoma (HepG2) cells in coculture based on morphological changes and Annexin-5 assays. Interestingly, a 72-h conditioned medium from hWJSCs (hWJSC-CM) also suppressed the growth of HepG2 tagged Green Fluorescent Protein (HepG2-GFP) cells *in vitro* (Fong CY et al, unpublished data). It thus appears that the anti-cancer effects of hWJSCs may be both cell-contact dependent as well as mediated via diffusible factors secreted by the hWJSCs. Based on the above published and unpublished observations, studies on the identification, isolation and characterization of putative anticancer agents from hWJSCs or hWJSC-CM would be very important and have tremendous clinical application.

Given these unique properties of hWJSCs we undertook a detailed transcriptome profiling of hWJSCs to shed some light as to whether there were any unusual expression of genes that confer on hWJSCs these remarkable characteristics. hWJSCs derived from 6 different umbilical cords (4 singletons and 1 set of fraternal twins) were subjected to Affymetrix DNA microarray analysis and the results compared with the known transcriptome profiles of hESCs, human embryonal carcinoma cells (hECCs), human fibroblast cells (hFCs) and hMSCs. We also determined whether the transcriptome profile of hWJSCs remains stable over extended passage. This is the first report of detailed transcriptome profiling of stem cells isolated specifically from the human Wharton's jelly that provides answers to some of its unique properties and lays the foundation for future confirmatory functional studies.

Materials and Methods

Derivation and Propagation of hWJSCs

Institutional Review Board (DSRB, National Healthcare Group, Singapore) approval was obtained for the collection of umbilical cords from human subjects by informed patient consent. hWJSCs were derived from 6 different umbilical cords, consisting of 4 singletons and 1 pair of fraternal twins (male, female) as previously described by our group [16]. The mid-region (7.5 cm) of each umbilical cord was collected in Hank's balanced salt solution (HBSS, Invitro-

gen, Carlsbad, CA) and stored at 4°C for 2–6 h before processing. Before derivation each piece was first washed in fresh HBSS and then cut into 1.5 cm pieces. Each small piece was slit open with scissors and the inner surface was exposed to a 1.5 ml enzymatic solution of collagenase type I, collagenase type IV and 100 IU of hyaluronidase (Sigma Chemical Co, USA) in DMEM medium (Invitrogen) in Petri dishes (100 mm) so as to allow only the Wharton's jelly to come into contact with the enzymes. The Petri dishes were incubated at 37°C in a 5% CO_2 in air atmosphere for 45 min to encourage loosening of the Wharton's jelly but not its complete digestion. After incubation, the cord pieces were transferred to new Petri dishes containing 3 ml of fresh DMEM medium to remove any traces of enzymes. Using the blunt surface of a pair of curved forceps the Wharton's jelly was gently separated into the fresh medium. The medium containing the Wharton's jelly was then collected into15 ml Falcon tubes and passed through an 18G needle using a syringe so as to release the hWJSCs. The suspension of hWJSCs was then collected into 15 ml Falcon tubes, centrifuged at 300 × g for 10 min, supernatant discarded and cell pellets resuspended in DMEM (high glucose) culture medium supplemented with 20% fetal bovine serum (FBS; Hyclone, Thermo Fisher Scientific Inc, Waltham, MA), 16 ng/ml bovine fibroblast growth factor (bFGF; Chemicon, Temecula, CA), 1 mML-glutamine, 1:200 dilution of insulin-transferrin-selenium (ITS) and antimycotic-antibiotic solution (Invitrogen). The cells and medium were seeded into T25 plastic tissue culture flasks (Becton Dickinson, BD, USA) and incubated at 37°C in a 5% CO_2 in air atmosphere. When cell confluence was reached in approximately 6–7 days, the monolayers were passaged with trypsin-EDTA (TryLE Express, Invitrogen). The hWJSC cultures were also routinely characterized for stem cell properties (flow cytometry CD markers and other stemness tests) and established to be multipotent as described in [16] and [27]. Representative morphological phenotypes of the hWJSC lines are shown in Fig 1.

Culture of hESC, hECC and hFC Lines

A karyotypically normal (HES3, ES Cell International Pte Ltd, Singapore) and a variant (BG01V; SCRC-2002, American Type Culture Collection (ATCC), Manassas, VA) hESC line were cultured on mitomycin-C treated mouse embryonic fibroblasts (MEFs) in bulk culture medium (BCM) composed of KnockOut DMEM medium (Invitrogen) supplemented with 20% KO serum replacement, 0.1 mM β-mercaptoethanol, 1 mML-glutamine, 0.1 nM nonessential amino acids, 50 Units/ml penicillin, 50 μg/ml streptomycin, 1:200 dilution of ITS (Invitrogen) and 16 ng/ml bFGF (Chemicon). Three hECC lines [NCCIT (CRL-2073, ATCC), NT2D1 (NTERA-2 cl.D1, CRL-1973, ATCC) and GCT27C4 (kind gift from Dr. Martin Pera)] and an hFC cell line [D551 (CCL-110, ATCC)] were cultured with RPMI 1640, DMEM (high glucose), DMEM/F12 and DMEM (Invitrogen) respectively. All culture media were supplemented with 10% FBS (Hyclone) and 1 mML-glutamine (Invitrogen).

Total RNA Extraction

Total RNA from the hESC cell lines and hWJSCs at early (3, 4, 5, 8) and late (20) passages were extracted using the RNeasy Mini Kit (QIAGEN GmbH, Hilden, Germany). Total RNA from the hECC and hFC cell lines were prepared using TRIzol reagent (Invitrogen) and purified with RNeasy MinElute Cleanup Kit (Qiagen). Total RNA yield was determined using NanoDrop™ 1000 (Thermo Fisher Scientific Inc) while RNA integrity was evaluated using the Agilent Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA).

Affymetrix GeneChip Human Genome U133 Plus 2.0 Array

The transcriptome profiles of the 3 classes of human stem cells: hESCs (HES3, BG01V); hECCs (NCCIT, NT2D1, GCT27C4); hWJSCs (4 singletons and 1 pair of fraternal

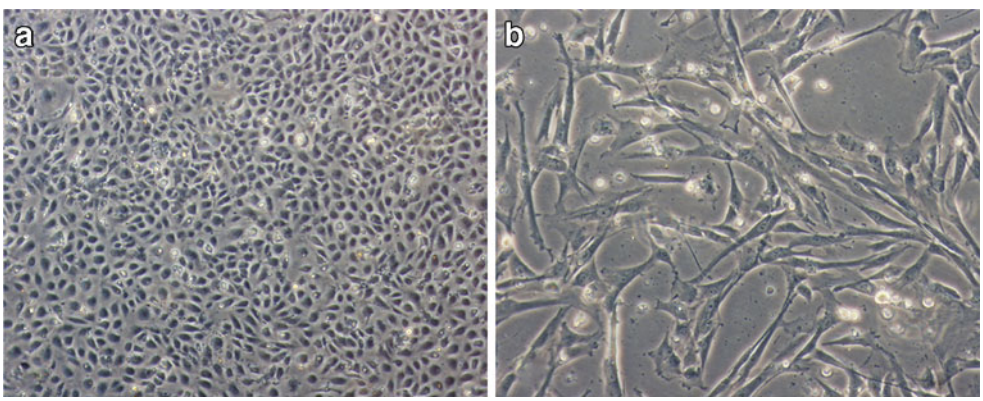


Fig. 1 Representative phase contrast images of primary and passaged hWJSC lines. **a** Primary culture of hWJSCs showing epitheliod-like morphology. Mag ×100. **b** Third passage hWJSC cultures showing fibroblastic-like morphology. Mag ×100

twins) and a normal fibroblast cell line (hFCs) (D551) were determined using the Affymetrix GeneChip Human Genome U133 plus 2.0 array (Affymetrix Inc, Santa Clara, CA). Briefly, first-strand synthesis was carried out with 3.5 μg of total RNA and the T7-Oligo(dT) primer at 42°C for 1 h. Second-strand reaction was performed at 16°C for 2 h. *In vitro* transcription to produce biotin-labeled cRNA was carried out at 37°C for 16 h. Purified biotin-labeled cRNA (20 μg) was fragmented at 94°C for 35 min. Hybridization was carried at 45°C, 60 rpm for 16 h in the Hybridization Oven 640 (Affymetrix). Washing of the GeneChip arrays was done on the Fluidics Station 450 (Affymetrix) while scanning was carried out with the GeneChip Scanner 3000. The Affymetrix GeneChip Operating Software (GCOS version 1.4) was used to obtain the CEL files containing the signal intensities from the scanned arrays. Quality control parameters for image quality, presence of artefacts, grid alignment, border staining and hybridization controls were also established prior to analysis.

Microarray Data Analysis

The Affymetrix Expression Console Software (version 1.0) was used for data processing. The summarized expression values were obtained using the Robust Multiarray Average (RMA) algorithm (parameters used were RMA Background Correction to PM-only probes and quantile normalization). The microarray data were deposited in NCBI Gene Expression Omnibus (GEO) under the accession number GSE20124 (Supplementary Table 1).

The GenePattern computational genomic environment (www.broadinstitute.org/genepattern) was used for data analysis [28]. Comparative analysis was done on a gene list that was selected through the Comparative Marker Selection module [29] with false discovery rate (FDR) of 5%. Two-sided t-test was performed with asymptotic *p*-value calculation and smoothening, and one-versus-all comparison for phenotype testing. FDR was estimated with the Benjamini-Hochberg procedure [30]. The conservative method of determining signal detection threshold with the hybridization control BioB (http://www.affymetrix.com/support/technical/manual/expression_manual.affx) which was spiked-in at the Affymetrix array, with a detection limit of 1.5 pM, was used to estimate the genes that were truly expressed above the experimental detection threshold [31]. Quartile tabulation of genes expressed above the detection threshold was performed to determine the expression sector of markers in each group [32].

Hierarchical clustering was performed using Pearson's correlation coefficient values and pairwise average-linkage clustering algorithm. Venn diagrams were drawn using the Venn diagram generator (http://www.pangloss.com/seidel/Protocols/venn.cgi). The GEO accession numbers for the additional microarray data that were used for meta-analysis are listed in Table 1. The entire dataset is available under GEO accession number GSE20125.

The Molecular Signatures Database (MSigDB) Gene Families [33] was used to determine the gene sets for cytokines, cell surface markers, tumour suppressor genes, and oncogenes. The stem cell and germ lineage markers were compiled from Cai et al. and Richards et al. [34, 35]. Markers for hMSCs, hHSCs and immune-associated genes were compiled from Anzalone et al. and La Rocca et al. [36, 37]. Gene Ontology (GO) Biological Process annotation of significantly regulated transcripts was performed with DAVID [38]. DAVID calculated EASE scores (modified Fisher Exact *P*-value) for GO Biological Process

Table 1 NCBI GEO accession numbers of samples used in the DNA microarray analyses*

Cell types	NCBI GEO Series	References
hWJSCs (human Wharton's jelly stem cells)	GSE20124	this paper
hESCs (human embryonic stem cells)	GSE20124	this paper
hECCs (human embryonal carcinoma cells)	GSE20124	this paper
hBMMSCs (human bone marrow mesenchymal stem cells)	GSE7888, GSE9451, GSE9520, GSE18043	[28–31]
hFCs (human fibroblast cells)	GSE9451, GSE9832, GSE20126	[29, 32], this paper
hUVECs (human umbilical vein endothelial cells)	GSE6241, GSE7307, GSE10479, GSE16683	[33, 34]
hUCBMSCs (human umbilical cord blood mesenchymal stem cells)	GSE13491	–
hATMSCs (human adipose mesenchymal stem cells)	GSE12843	[35]
hLPMSCs (human lipoma mesenchymal stem cells)	GSE12843	[35]
hTEMSCs (human teeth mesenchymal stem cells)	GSE15214	[36]
hTS (human tumour stroma: B, breast; P, prostate; BL, bladder)	GSE8977, GSE17906	[37, 38]
hNS (human normal stroma: B, breast; P, prostate; BL, bladder)	GSE8977, GSE17906	[37, 38]

* Please see Supplementary Table 1 for full sample details

associated with death, immune system, and chemotaxis were used for the heat map visualization and generated using the Heat map Viewer (GenePattern). To enable gradient distinction between the EASE scores missing values were replaced with a value of 0.1.

Quantitative Real-time PCR

First-strand cDNA synthesis was carried out with 2.5 μg of total RNA with the SuperScript™ III First-Strand Synthesis System for RT-PCR (Invitrogen) and random hexamers. Relative quantitative real-time PCR (qRT-PCR) was performed in triplicates using the ABI 7500 Fast System (Applied Biosystems, Foster City, CA) for 50 cycles with a 10 μl reaction volume. Each reaction contained 20 ng of cDNA, 4 μM forward/reverse primer mix, 1 μM probe from the Roche Universal Probe Library (UPL; Roche Applied Science, Indianapolis, IN), and Taqman Universal PCR Master Mix (Applied Biosystems). The PCR primers and UPL Probes used are presented in Table 2.

Data analysis was carried out using SDS 7500 v2.0 Software (Applied Biosystems). The cycle threshold (*Ct*) was determined with automatic baseline calculations and outliers (SD≥0.3) were then removed prior to the calculation of the average *Ct* values and were exported into Microsoft Excel for further analysis. Normalization was carried out with the 18S rRNA *Ct* value as follows, $\Delta Ct = Ct$ [gene]—*Ct*[18S]. The mean *Ct* value for 18S rRNA in all samples was 8. As the limit of detection of qRT-PCR (cycle cut-off) was determined to be around *Ct* value of 35, ΔCt value above 27 was considered to be undetected.

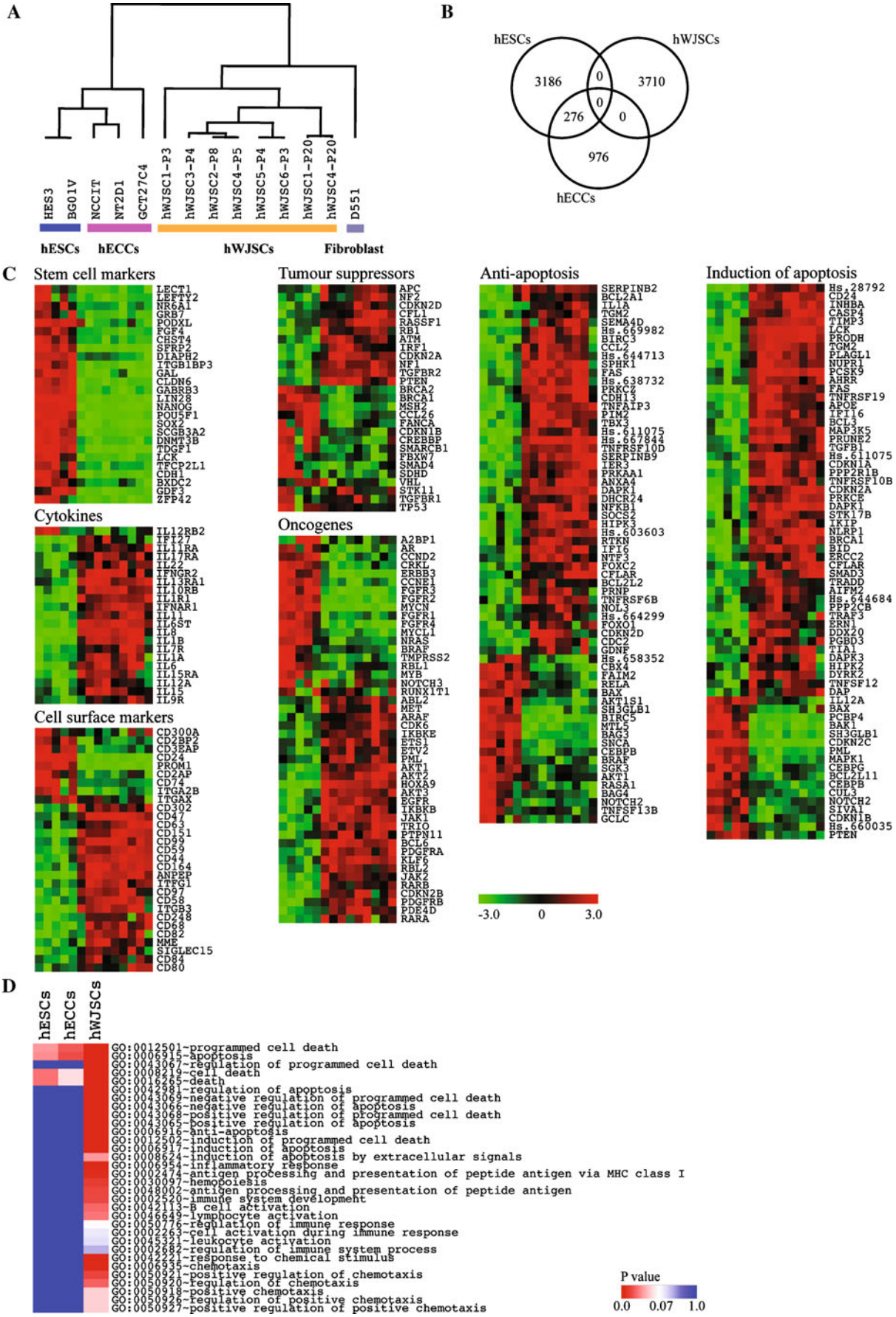
Results

Based on Genepattern Software and Pearson's Correlation Coefficients hWJSCs are a Unique Class of MSCs with Very Low Expression Levels of ESC Markers

Using GenePattern Software, global differences in the transcriptomes were observed between the 3 classes of stem cells (hESC, hECC, hWJSC) and the fibroblast cell line (hFC) (Fig. 2). When the upregulated genes (FDR 5%) for each class of stem cells were examined, they could be combined to produce an upregulated dataset comprising of 8148 genes. Hierarchical clustering analysis revealed that the hWJSCs (irrespective of the number of passages) were tightly clustered together, while the hESCs and hECCs forming a distinct separate cluster (Fig. 2a). Additionally, the Pearson's correlation coefficient values among the early-passage hWJSCs ranged from 0.952 to 0.988, with the hWJSCs from the fraternal twins showing the highest correlation (0.988). This indicates that the gene expression profile among the hWJSCs was nearly invariable (Supplementary Table 2). The lowest correlation coefficient was consistent for comparisons involving the hWJSC1-passage

Table 2 List of primers and UPL probes used for real-time PCR

Gene name	RefSeq	Sequences	UPL No
POU5F1 variant 1 (OCT4A)	NM_002701.4	F: 5′-GTGGAGAGCAACTCCGATG-3′ R: 5′-TCTGCAGAGCTTTGATGTCC-3′	78
SOX2	NM_003106.2	F: 5′-TTGCTGCCTCTTTAAGACTAGGA-3′ R: 5′-CTGGGGCTCAAACTTCTCTC-3′	35
NANOG	NM_024865.2	F: 5′-AGATGCCTCACACGGAGACT-3′ R: 5′-TTTGCGACACTCTTCTCTGC-3′	31
BIRC3	NM_001165.3, NM_182962.1	F: 5′-GACTGGGCTTGTCCTTGCT-3′ R: 5′-AAGAAGTCGTTTTCCTCCTTTGT-3′	44
BIRC5	NM_001168.2, NM_001012270.1, NM_001012271.1	F: 5′-CACCGCATCTCTACATTCAAGA-3′ R: 5′-CAAGTCTGGCTCGTTCTCAGT-3′	86
IL1B	NM_000576.2	F: 5′-CTGTCCTGCGTGTTGAAAGA-3′ R: 5′-TTGGGTAATTTTTGGGATCTACA-3′	78
IL6	NM_000600.2	F: 5′-GATGAGTACAAAAGTCCTGATCCA-3′ R: 5′-CTGCAGCCACTGGTTCTGT-3′	40
IL6ST	NM_002184.2, NM_175767.1	F: 5′-GTTCTGCTTTAATAAGCGAGACCT-3′ R: 5′-ATTGTGCCTTGGAGGAGTGT-3′	61
IL8	NM_000584.2	F: 5′-AGACAGCAGAGCACACAAGC-3′ R: 5′-ATGGTTCCTTCCGGTGGT-3′	72
IL12A	NM_000882.2	F: 5′-CACTCCCAAAACCTGCTGAG-3′ R: 5′-TCTCTTCAGAAGTGCAAGGGTA-3′	50


A
HES3
BG01V
NCCIT
NT2D1
GCT27C4
hWJSC1-P3
hWJSC3-P4
hWJSC2-P8
hWJSC4-P5
hWJSC5-P4
hWJSC6-P3
hWJSC1-P20
hWJSC4-P20
D551
hESCs
hECCs
hWJSCs
Fibroblast
B
hESCs
hWJSCs
hECCs
3186
0
3710
0
276
0
976
C
Stem cell markers
LECT1
LEFTY2
NR6A1
GRB7
PODXL
PGF4
CHST4
SFRP2
DIAPH2
ITGB1BP3
GAL
CLDN6
GABRB3
LIN28
NANOG
POU5F1
SOX2
SCGB3A2
DNMT3B
TDGF1
LCK
TFCP2L1
CDH1
BXDC2
GDF3
ZFP42
Tumour suppressors
APC
NF2
CDKN2D
CFL1
RASSF1
RB1
ATM
IRF1
CDKN2A
NF1
TGFBR2
PTEN
BRCA2
BRCA1
MSH2
CCL26
FANCA
CDKN1B
CREBBP
SMARCB1
FBXW7
SMAD4
SDHD
VHL
STK11
TGFBR1
TP53
Anti-apoptosis
SERPINB2
BCL2A1
IL1A
TGM2
SEMA4D
Hs.669982
BIRC3
CCL2
Hs.644713
SPHK1
FAS
Hs.638732
PRKCZ
CDH13
TNFAIP3
PIM2
TBX3
Hs.611075
Hs.667844
TNFRSF10D
SERPINB9
IER3
PRKAA1
ANXA4
DAPK1
DHCR24
TP53I3
NFKB1
SOCS2
HIPK3
Hs.603603
RTKN
IFIG
NTF3
FOXC2
CFLAR
BCL2L2
PRNP
TNFRSF6B
NOL3
Hs.664299
FOXO1
CDKN2D
CDC2
GDNF
Hs.658352
CBX4
FAIM2
RELA
BAX
AKT1S1
SH3GLB1
BIRC5
MTL5
BAG3
SNCA
CEBPB
BRAF
SGK3
AKT1
RASA1
BAG4
NOTCH2
TNFSF13B
GCLC
Induction of apoptosis
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INHBA
CASP4
TIMP3
LCK
PRODH
TGM2
PLAGL1
NUPR1
PCSK9
AHRR
FAS
TNFRSF19
APOE
IFI16
BCL3
MAP3K5
PRUNE2
TGFB1
Hs.611075
CDKN1A
PPP2R1B
TNFRSF10B
CDKN2A
PRKCE
DAPK1
STK17B
IKIP
NLRP1
BRCA1
BID
ERCC2
CFLAR
SMAD3
TRADD
AIFM2
Hs.644684
PPP2CB
TRAF3
ERN1
DDX20
PGBD3
TIA1
DAPK3
HIPK2
DYRK2
TNFSF12
DAP
IL12A
BAX
PCBP4
BAK1
SH3GLB1
CDKN2C
PML
MAPK1
CEBPG
BCL2L11
CEBPB
CUL3
NOTCH2
SIVA1
CDKN1B
Hs.660035
PTEN
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0
3.0
Cytokines
IL12RB2
IFI27
IL1RA
IL17RA
IL22
IFNGR2
IL13RA1
IL10RB
IL1R1
IFNAR1
IL11
IL6ST
IL8
IL1B
IL7R
IL1A
IL6
IL15RA
IL12A
IL15
IL9R
Oncogenes
A2BP1
AR
CCND2
CRKL
ERBB3
CCNE1
FGFR3
FGFR2
MYCN
FGFR1
FGFR4
MYCL1
NRAS
BRAF
TMPRSS2
RBL1
MYB
NOTCH3
RUNX1T1
ABL2
MET
ARAF
CDK6
IKBKE
ETS1
ETV2
PML
AKT1
AKT2
HOXA9
AKT3
EGFR
IKBKB
JAK1
TRIO
PTPN11
BCL6
PDGFRA
KLF6
RBL2
JAK2
RARB
CDKN2B
PDGFRB
PDE4D
RARA
Cell surface markers
CD300A
CD2BP2
CD3EAP
CD24
PROM1
CD2AP
CD74
ITGA2B
ITGAX
CD302
CD47
CD63
CD151
CD99
CD59
CD44
CD164
ANPEP
ITFG1
CD97
CD58
ITGB3
CD248
CD68
CD82
MME
SIGLEC15
CD84
CD80
D
hESCs
hECCs
hWJSCs
GO:0012501~programmed cell death
GO:0006915~apoptosis
GO:0043067~regulation of programmed cell death
GO:0008219~cell death
GO:0016265~death
GO:0042981~regulation of apoptosis
GO:0043069~negative regulation of programmed cell death
GO:0043066~negative regulation of apoptosis
GO:0043068~positive regulation of programmed cell death
GO:0043065~positive regulation of apoptosis
GO:0006916~anti-apoptosis
GO:0012502~induction of programmed cell death
GO:0006917~induction of apoptosis
GO:0008624~induction of apoptosis by extracellular signals
GO:0006954~inflammatory response
GO:0002474~antigen processing and presentation of peptide antigen via MHC class I
GO:0030097~hemopoiesis
GO:0048002~antigen processing and presentation of peptide antigen
GO:0002520~immune system development
GO:0042113~B cell activation
GO:0046649~lymphocyte activation
GO:0050776~regulation of immune response
GO:0002263~cell activation during immune response
GO:0045321~leukocyte activation
GO:0002682~regulation of immune system process
GO:0042221~response to chemical stimulus
GO:0006935~chemotaxis
GO:0050921~positive regulation of chemotaxis
GO:0050920~regulation of chemotaxis
GO:0050918~positive chemotaxis
GO:0050926~regulation of positive chemotaxis
GO:0050927~positive regulation of positive chemotaxis
P value
0.0
0.07
1.0

◀ **Fig. 2** Microarray analysis of human Wharton's jelly stem cells. **a** Hierarchical clustering showed the relationships of human embryonic stem cells (hESCs), human embryonal carcinoma cells (hECCs), human Wharton's jelly stem cells (hWJSCs), and human fibroblast cells (hFCs) (D551). The hWJSCs were grouped tightly together with Pearson's correlation coefficient values showing close but distinct relationship between the early and late passage hWJSCs. **b** hWJSCs showed a distinct profile of differentially expressed genes compared to hESCs and hECCs. A large number of genes that were significantly expressed in both the hESCs and hECCs belonged to embryonic stem cell (ESC) markers. **c** hWJSCs showed distinct gene expression patterns for ESC markers, cytokines, cell surface markers, oncogenes and tumour suppressor genes as compared to hESCs, hECCs and hFCs (D551). In particular, hWJSCs had high expression of specific cytokines and carried specific cell surface markers. **d** hWJSCs were significantly associated with GO Biological Process terms related to cell death and the immune system. For instance, hWJSCs showed distinctive patterns of upregulated genes associated with anti-apoptosis and induction of apoptosis as compared to hESCs and hECCs

3 (hWJSC1-P3). Nevertheless, the 2 late passages (P20) for hWJSC1 and hWJSC4 were very similar, indicating that the gene expression differences in hWJSC1-P3 were minimized through extended passages. Gender difference appeared to exert a slight influence as well. Moreover, Pearson's correlation coefficient values showed that there were little differences in gene expression profile between early passages and late passages (Supplementary Table 2) and they could be accounted for by culture conditions. This confirms that the isolation process for hWJSCs is robust [16] and a relatively pure population of hWJSCs could be readily isolated with little discernible differences in their transcriptome profiles even after extended culture.

A three-way Venn diagram of hESC (3462 genes), hECC (1252 genes), and hWJSC (3710 genes) was drawn with the statistically significant genes (FDR 5%) that were upregulated in one class versus the rest (Fig. 2b). The major gene symbols with their names are shown in Table 3. All the significantly upregulated genes in hWJSCs showed no overlap between hESCs or hECCs (Supplementary Table 3). This suggested that the hWJSCs were significantly different from hESCs and hECCs. The top 10 genes with the highest fold change and upregulated in hWJSCs (compared to hESCs and hECCs) were IL8, SRGN, IL33, GBP3, PTGS2, CXCL1, NT5E, CFH/CFHR1, SEMA3C, and TNFRSF11B. Among these genes, IL8, CFHR1, CXCL1, and SEMA3C are associated with Gene Ontology Biological Process terms for chemotaxis and the immune system response. The hESCs and hECCs however shared 276 significantly upregulated genes and within this overlap were a large number of embryonic stem cell markers which included POU5F1, SOX2, NANOG, and LIN28, that are important for stem cell maintenance and the induction of pluripotency (Supplementary Table 4).

Microarray Confirms that hWJSCs Coexpress MSC Markers while ESC Markers are Barely Detected

The GSEA MSigDB was used to analyze the expression profile of selected gene families (Fig. 2c). To enhance robustness of our analyses of the Affymetrix DNA microarray data we adopted a stringent detection limit for signal intensities. Many of the stem cell makers that were highly expressed in hESCs and hECCs, which included POU5F1, NANOG, SOX2, LIN28, TDGF1, PODXL, DNMT3B, CLDN6, DIAPH2, GABRB3 and SCGB3A2 [34, 35] were not detected or had very low expression in hWJSCs. Only ITGB1, IFITM2, CD9, GJA1, and IGF2BP2 that are highly expressed in hESCs, hECCs, and hMSCs were detected for the hWJSCs. hWJSCs also express the stem cell associated markers IL6ST, PTEN, and COMMD3, which were found in hESCs and hBMMSCs [39, 40]. What was significant was that hWJSCs showed a distinctive high expression profile of cytokines and cell surface markers compared to hESCs and hECCs. Indeed, the most differentially up-regulated gene in hWJSCs was IL8, with a 30 fold difference with other notable upregulated cytokines being IL1B, IL6, IL6ST, and IL12A.

hWJSCs showed high expression of various MSC markers such as CD44, NT5E (CD73), THY1 (CD90), ITGB1 (CD29), CD44, ENG (CD105), ALCAM (CD166), ANPEP (CD13) and MME (CD10). They did not express key hematopoietic and endothelial markers such as PECAM1 (CD31), CD33, CD34, CD45, VWF, CDH5, ICAM2, KDR, and FLT1. The high expression of the endothelial markers, EPAS1, EDF1, VEGFA, and VEGFC, and low expression of AAMP, ECE1, and TEK seen in our hWJSCs are consistent with other reports [41]. In addition, hWJSCs do not express markers for circulating fibrocytes (HLA-DR) [42] or lineage markers for monocyte or macrophage-like CD14, ITGAM (CD11b), ITGB2 (CD18), and CSF1R (CD115). hWJSCs expressed high levels of the HLA MHC Class 1 genes (HLA-A, B, C, E, F, G), but HLA MHC Class 2 genes were well below the detection level [37]. In conclusion, the lack of expression of lineage-specific markers showed that the hWJSCs were maintained as a relatively undifferentiated and pure MSC population, with little or no contamination with other cell types.

The expression profiles of oncogenes and tumour suppressors in hESCs, hECCs and hWJSCs were also analyzed (Fig. 2c). hWJSCs expressed a completely different subset of oncogenes and tumour suppressor genes from hESCs and hECCs. The tumour suppressor genes TGFBR2, CDKN2A, ATM, NF1, PTEN, and CFL1 were upregulated in hWJSCs compared to hESCs and hECCs and the highest expressed oncogene and tumour suppressor genes in hWJSCs were PDGFRA (4-fold) and TGFBR2 (6-

Table 3 Gene symbols and names of major genes

AAMP	Angio-associated, migratory cell protein
ACTA2	Actin alpha 2
ALCAM (CD166)	Activated leukocyte cell adhesion molecule
ANPEP (CD13)	Alanine aminopeptidase
ATM	Ataxia telangiectasia mutated
BIRC5	Survivin
CD14	CD14
CD33	CD33
CD34	CD34
CD44	CD44
CD45	Protein tyrosine phosphatase, receptor type, C
CD9	CD9
CDH5	Cadherin 5, type 2 or VE-cadherin (vascular endothelial), CD 144
CDKN2A	Cyclin-dependent kinase inhibitor 2A
CFH/CFHR1	Complement Factor H / Complement factor H-related protein 1
CFL1	Cofilin 1 (non-muscle; n-cofilin)
CLDN6	Claudin-6
COL1A1	Collagen, type I, alpha 1
COMMD3	COMM domain-containing protein 3
CSF1R (CD115)	Colony stimulating factor 1 receptor
CXCL1	Chemokine (C-X-C motif) ligand 1
DIAPH2	Protein diaphanous homolog 2
DNMT3B	DNA (cytosine-5-)-methyltransferase 3 beta
ECE1	Endothelin converting enzyme 1
EDF1	Endothelial differentiation-related factor 1
ENG (CD105)	Endoglin
EPAS1	Endothelial PAS domain-containing protein 1
FLT1	Vascular endothelial growth factor receptor 1
FN1	Fibronectin
FOXD3	Forkhead box D3
GABRB3	Gamma-aminobutyric acid receptor subunit beta-3
GATA6	GATA binding protein 6
GBP3	Guanylate-binding protein 3
GJA1	Gap junction alpha-1 protein
ICAM2	Intercellular adhesion molecule 2 (ICAM2) (CD102)
IFITM2	Interferon induced transmembrane protein 2
IGF2BP2	Insulin-like growth factor 2 mRNA-binding protein 2
IL12A	Interleukin-12 subunit alpha
IL1B	Interleukin 1, beta
IL33	Interleukin 33
IL6	Interleukin-6
IL6ST	Glycoprotein 130 (CD 130)
IL8	Interleukin-8
ITGAM (CD11b)	Integrin alpha M
ITGB1 (CD29)	Integrin beta-1
ITGB2 (CD18)	Integrin beta-2
KDR	Kappa Delta Rho
LAMC1	Laminin subunit gamma-1
LIN28	Lin-28 homolog A
MAP2	Microtubule-associated protein 2
MME (CD10)	Membrane metallo-endopeptidase (Neprilysin)
NANOG	Nanog
NES	Nestin
NEUROD1	Neurogenic differentiation 1
NF1	Neurofibromin 1
NSE	Enolase 2 (gamma, neuronal), neuron-specific enolase
NT5E (CD73)	5′-nucleotidase, ecto
NUMB	Protein numb homolog
PDGFRA	Alpha-type platelet-derived growth factor receptor
PECAM1 (CD31)	Platelet/endothelial cell adhesion molecule
PMAIP1	Phorbol-12-myristate-13-acetate-induced protein 1
PODXL	Podocalyxin-like protein 1
POU5F1	Oct-4
PTEN	Phosphatase and tensin homolog
PTGS2	Prostaglandin-endoperoxide synthase 2
SCGB3A2	Secretoglobin family 3A member 2
SEMA3A	Semaphorin-3A
SEMA3C	Semaphorin-3C
SMAD2	SMAD family member 2
SOX1	Sex determining region Y-box 1
SOX17	SRY (sex determining region Y)-box 17
SOX2	SRY (sex determining region Y)-box 2
SRGN	Serglycin
TDGF1	Teratocarcinoma-derived growth factor 1
TEK	Angiopoietin-1 receptor
TGFBR2	Transforming growth factor, beta receptor II
THY1 (CD90)	Thymocyte differentiation antigen 1
TNFRSF11B	Osteoprotegerin (OPG)
TUBB3	Tubulin beta-3 chain
VEGFA	Vascular endothelial growth factor A (VEGF-A)
VEGFC	Vascular endothelial growth factor C
VWF	von Willebrand factor

fold) respectively, with no expression for these genes detected in both hESCs and hECCs.

hWJSCs are Associated with Apoptosis and Immune System Regulation

Gene ontology annotation was performed with DAVID using the significantly up-regulated gene list (FDR 5%). Based on the EASE scores, hWJSCs had very high

association with cell death, immune system and chemotaxis related GO Biological Process terms compared to hESCs and hECCs (Fig. 2d; Supplementary Table 5) indicating that the expression of a large number of genes associated with these biological processes were expressed in hWJSCs. hWJSCs also expressed a significantly different and greater subset of genes associated with anti-apoptosis and induction of apoptosis compared to hESCs and hECCs (Fig. 2c). The expression levels of these genes were also higher in hWJSCs compared to hESCs and hECCs and IL12A which is associated with induction of apoptosis was among the upregulated cytokines. BIRC5 was expressed at lower levels in hWJSCs compared to hESCS and hECCs and interestingly BIRC5 which is an anti-apoptotic gene associated with cell survival was recently shown to be required for hESC-induced *in vivo* teratoma formation in SCID mice [43].

Meta-analysis of hWJSCs, Adult Stem Cells and Stromal Cells

Meta-analyses of DNA microarray data pooled from different studies have been used to determine gene expression signatures of specific cell types [32, 44, 45]. Using a similar approach, the relationships and expression profiles were compared between the hWJSC Affymetrix microarray data and human umbilical cord blood mesenchymal stem cells (hUCBMSCs) (fetal), human umbilical vein endothelial cells (hUVECs) (fetal), human bone marrow mesenchymal stem cells (hBMMSCs) (adult) and human normal stroma (hNS), human tumour stroma (hTS), human fibroblast cells (hFC) (human stromal cells) (Table 1). Human umbilical vein endothelial cells (hUVECs) were used for comparison as a control non-stem cell population lying within the human umbilical cord. The final dataset (FDR 5%) contained 8627 genes that were upregulated in at least one class. Since human adipose mesenchymal stem cells (hATMSCs), human lipoma mesenchymal stem cells (hLPMSCs), and human teeth mesenchymal stem cells (hTEMSCs) (Table 1) did not contain biological replicates, they were used for clustering analysis but not included in the statistical analysis.

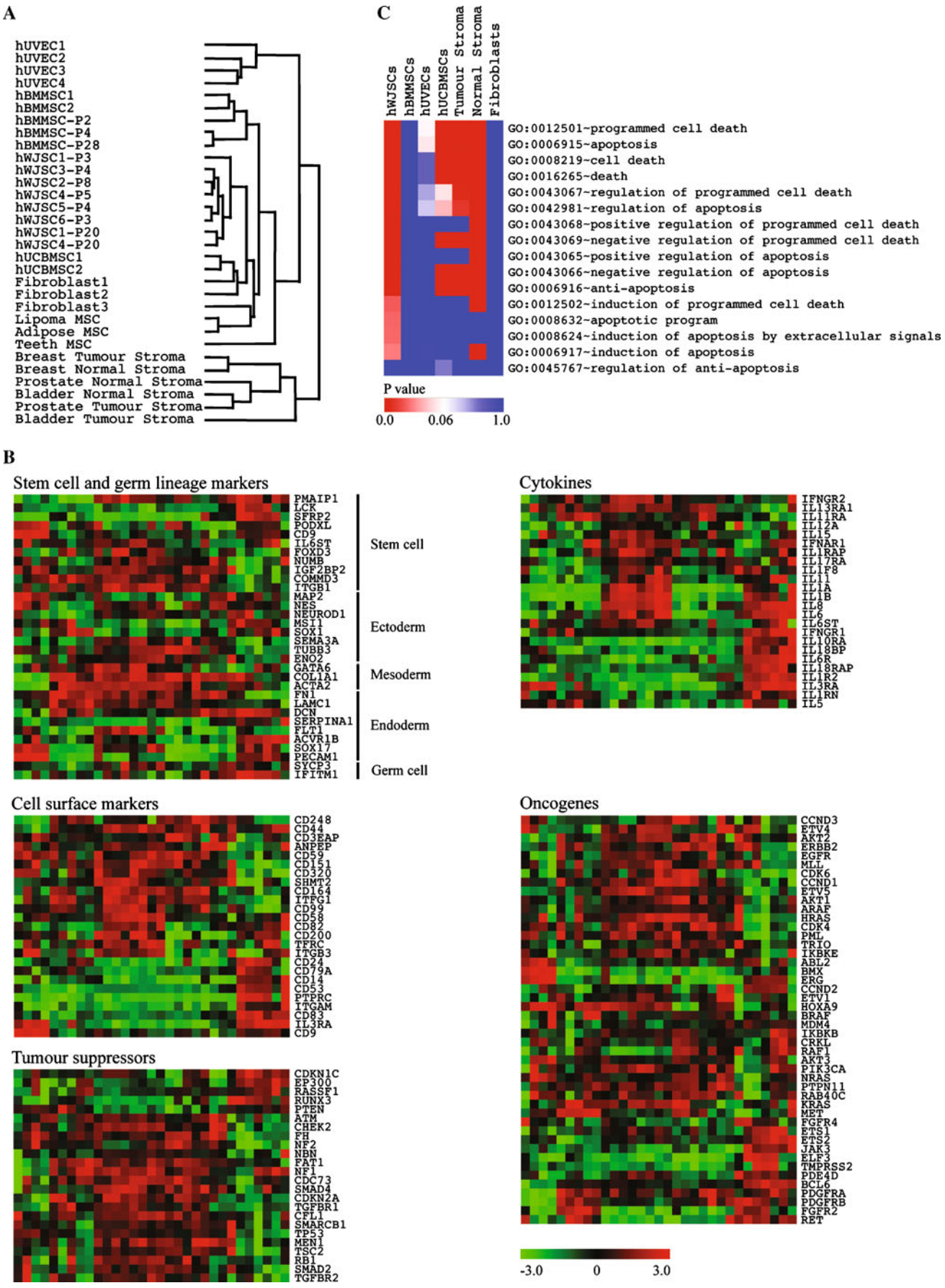
Hierarchical clustering showed that the samples grouped within their class type despite being from different studies (Fig. 3a) indicating that the analysis method was appropriate. Two main groups were formed in the hierarchical clustering viz., MSCs and stromal cells. The hWJSCs grouped closest to hUCBMSCs and this was perhaps because of their close origin in the human umbilical cord. Interestingly, the hUVECs grouped away from the hWJSCs and hUCBMSCs which were probably due to differentiation of hUVECs to the endothelial lineage compared to the other less lineage-committed MSCs.

hWJSCs Have a Wider Range of Expression of Stem Cell and Lineage Markers Compared to other MSCs

hWJSCs had a wider range of stem cell and lineage markers expressed compared to other fetal and adult MSCs and stromal cells (Fig. 3b). The stem cell markers that were significantly upregulated in hWJSCs were ITGB1, PMAIP1, IGF2BP2, NUMB, FOXD3, and SMAD2. Among the fetal and adult stem cells in this meta-analysis only hWJSCs had upregulated markers for all 3 primordial germ layers. hWJSCs showed an upregulation of markers for ectoderm (SEMA3A, NEUROD1, NES, SOX1, NSE), endoderm (LAMC1, FN1), and mesoderm (ACTA2, GATA6, COL1A1); while hBMMSCs had upregulation only for ectoderm (SOX1) and endoderm (LAMC1, FN1); hUCBMSCs had upregulated markers for ectoderm (TUBB3), mesoderm (COL1A1) and the stem cell associated markers (ITGB1, PODXL); and hUVECs had upregulation for ectoderm (MAP2), endoderm (SOX17), and the stem cell associated markers (PODXL, PTEN, CD9).

There were distinct gene expression patterns between hWJSCs and the other classes of MSCs for the selected gene families (Fig. 3b). hWJSCs had the highest number of significantly expressed cytokines and cell surface markers. There were 14 significantly upregulated cytokines in hWJSCs (including IL8, IL6, IL1B, IL12A) compared to only 2 (IL11RA and IL3RA) in hBMMSCs and hUVECs. In comparison, hNS and hTS had 7 upregulated cytokines each. Similarly, hWJSCs had 13 upregulated cell surface markers while there was only 1 each for hBMMSCs and hUVECs, and 3 for hUCBMSCs. As expected, the hUVECs had high expression of the endothelial markers CDH5, ICAM2, TEK, KDR, VWF and PECAM1. Expression of the HLA MHC Class 1 genes was found in all adult and fetal stem cells while hWJSCs had significant upregulation for HLA-G.

There are 54 tumour suppressor genes and 99 oncogenes annotated in the GSEA MSigDB Gene families [33]. Out of these, hWJSCs and hUCBMSCs expressed the highest number of upregulated tumour suppressor genes and oncogenes and hWJSCs also had more upregulated tumour suppressor genes than hUCBMSCs (Fig. 3b). There were a similar number of oncogenes for hWJSCs and hUCBMSCs with 10 oncogenes shared between them. The *P*-value heat map of GO Biological Process terms indicated that hWJSCs had the largest number of significant terms for pro-survival and pro-apoptosis. Within these categories, hWJSCs were enriched with terms associated with induction of apoptosis in contrast with other MSCs (Fig. 3c; Supplementary Table 6). This is reflective of the high cytokine expression profile seen for hWJSCs (Fig. 3b).


A
hUVEC1
hUVEC2
hUVEC3
hUVEC4
hBMMSC1
hBMMSC2
hBMMSC-P2
hBMMSC-P4
hBMMSC-P28
hWJSC1-P3
hWJSC3-P4
hWJSC2-P8
hWJSC4-P5
hWJSC5-P4
hWJSC6-P3
hWJSC1-P20
hWJSC4-P20
hUCBMSC1
hUCBMSC2
Fibroblast1
Fibroblast2
Fibroblast3
Lipoma MSC
Adipose MSC
Teeth MSC
Breast Tumour Stroma
Breast Normal Stroma
Prostate Normal Stroma
Bladder Normal Stroma
Prostate Tumour Stroma
Bladder Tumour Stroma
C
hWJSCs
hBMMSCs
hUVECs
hUCBMSCs
Tumour Stroma
Normal Stroma
Fibroblasts
GO:0012501~programmed cell death
GO:0006915~apoptosis
GO:0008219~cell death
GO:0016265~death
GO:0043067~regulation of programmed cell death
GO:0042981~regulation of apoptosis
GO:0043068~positive regulation of programmed cell death
GO:0043069~negative regulation of programmed cell death
GO:0043065~positive regulation of apoptosis
GO:0043066~negative regulation of apoptosis
GO:0006916~anti-apoptosis
GO:0012502~induction of programmed cell death
GO:0008632~apoptotic program
GO:0008624~induction of apoptosis by extracellular signals
GO:0006917~induction of apoptosis
GO:0045767~regulation of anti-apoptosis
P value
0.0
0.06
1.0
B
Stem cell and germ lineage markers
PMAIP1
LCK
SFRP2
PODXL
CD9
IL6ST
FOXD3
NUMB
IGF2BP2
COMMD3
ITGB1
MAP2
NES
NEUROD1
MSI1
SOX1
SEMA3A
TUBB3
ENO2
GATA6
COL1A1
ACTA2
FN1
LAMC1
DCN
SERPINA1
FLT1
ACVR1B
SOX17
PECAM1
SYCP3
IFITM1
Stem cell
Ectoderm
Mesoderm
Endoderm
Germ cell
Cytokines
IFNGR2
IL13RA1
IL11RA
IL12A
IL15
IFNAR1
IL1RAP
IL17RA
IL1F8
IL11
IL1A
IL1B
IL8
IL6
IL6ST
IFNGR1
IL10RA
IL18BP
IL6R
IL18RAP
IL1R2
IL3RA
IL1RN
IL5
Cell surface markers
CD248
CD44
CD3EAP
ANPEP
CD59
CD151
CD320
SHMT2
CD164
ITFG1
CD99
CD58
CD82
CD200
TFRC
ITGB3
CD24
CD79A
CD14
CD53
PTPRC
ITGAM
CD83
IL3RA
CD9
Oncogenes
CCND3
ETV4
AKT2
ERBB2
EGFR
MLL
CDK6
CCND1
ETV5
AKT1
ARAF
HRAS
CDK4
PML
TRIO
IKBKE
ABL2
BMX
ERG
CCND2
ETV1
HOXA9
BRAF
MDM4
IKBKB
CRKL
RAF1
AKT3
PIK3CA
NRAS
PTPN11
RAB40C
KRAS
MET
FGFR4
ETS1
ETS2
JAK3
ELF3
TMPRSS2
PDE4D
BCL6
PDGFRA
PDGFRB
FGFR2
RET
Tumour suppressors
CDKN1C
EP300
RASSF1
RUNX3
PTEN
ATM
CHEK2
FH
NF2
NBN
FAT1
NF1
CDC73
SMAD4
CDKN2A
TGFBR1
CFL1
SMARCB1
TP53
MEN1
TSC2
RB1
SMAD2
TGFBR2
-3.0
0
3.0

◀ **Fig. 3** Meta-analysis of transcriptome profiles of hWJSCs, human mesenchymal stem cells (hMSCs), hFCs and stromal cells. **a** Adult stem cells and hWJSCs clustered according to cell type regardless of their origin, while stromal cells formed their own distinctive cluster. The hMSCs that clustered closest to hWJSCs were human umbilical cord blood mesenchymal stem cells (hUCBMSCs). **b** hWJSCs showed distinct gene expression patterns for specific gene families compared to the adult hMSCs and stromal cells with its closest counterpart in hUCBMSCs. Importantly, hWJSCs had the highest expression of stem cell and germ lineage markers among the hMSCs. Cell surface marker and cytokine expression were also distinctive in the hWJSCs. **c** hWJSCs were highly associated with death-related GO Biological Process terms. Interestingly, genes associated with the induction of apoptosis were especially upregulated in hWJSCs

Real-time PCR Validation of Microarray Gene Expression

Our Affymetrix DNA microarray results indicated that pluripotency-associated markers showed low level expression in hWJSCs while some studies have reported higher levels of expression of POU5F1, NANOG and SOX2 in human or porcine WJSCs using real-time PCR or RT-PCR [16, 37, 40, 46, 47]. Therefore we carried out qRT-PCR on the hWJSCs of the present study to validate the expression levels of POU5F1, NANOG, and SOX2 across a larger panel of hWJSCs (Fig. 4a). Although the expression of POU5F1, NANOG, and SOX2 in both early and late passage hWJSCs could be detected by qRT-PCR, they were still extremely low, with mean *Ct* values of about 1000- to 1500-fold lower compared to pluripotent hESCs. The *Ct* values obtained for all the hWJSC lines were in fact just above the threshold for the limit of detection of the qRT-PCR assays with the exception of hWJSC2-P8 whose expression of POU5F1, NANOG, and SOX2 was about 10-fold higher than the other hWJSCs which is still very much lower than that for hESCs. The mean expression of these 3 pluripotency factors was also consistently lower in the late passages of hWJSCs.

Real-time PCR to determine the expression levels for the cytokines, IL1B, IL6ST, IL6, IL8, and IL12A (Fig. 4b), and 2 anti-apoptosis genes (BIRC3 and BIRC5) were also carried out (Fig. 4a). While slight variations in expression level among the hWJSCs were evident, in general the qRT-PCR results correlated well with the DNA microarray data. The expression of IL6ST and IL1B were stronger than IL6 and IL8 and the mean expression for the cytokines and anti-apoptosis genes were not significantly different between early and late passage hWJSCs.

Discussion

Robust Derivation and Propagation Procedures for hWJSCs Yields Homogeneous MSC Populations

For this study we used the Affymetrix Human Genome U133 Plus 2.0 DNA microarray which is capable of detecting ~38,500 human genes, to determine the transcriptome profiles of hWJSCs according to our derivation protocol [16]. The gene expression profiles of all early passage hWJSCs were very similar as revealed by the high Pearson's correlation coefficient values based on pair-wise comparison of their transcriptome profiles. In addition, the DNA microarray analysis showed strong expression of MSC markers in the hWJSCs with no evidence of the presence of other contaminating cell types.

It has been reported that circulating fetal fibrocytes and macrophages could migrate into the Wharton's jelly from the umbilical vessels [42]. However, markers for fetal fibrocytes (HLA-DR, CD163, CD45, CXCR4), macrophages and monocytes [CD14, ITGAM (CD11b), ITGB2 (CD18), CSF1R (CD115)] were not detected in the hWJSCs. Other MSCs observed in the human umbilical cord are in the cord blood and vein endothelial cells, and similarly, expression of key markers for these cells [VWF, CDH5, ICAM2, PECAM1 (CD31), KDR, FLT1, CD33, CD45, and CD34] was also not found in hWJSCs. The expression profile we observed for endothelial markers was consistent with previously published results [41]. Therefore taken together, transcriptome profiling confirmed that the hWJSCs derived via the protocol we have described [16] yields a highly homogeneous population of stem cells. The transcriptome profiles did not change even after 20 passages confirming the stability of the cells in the described derivation and propagation culture protocol [16].

hWJSCs Exhibit Wide Multipotent Stemness Properties

In the present study, we describe for the first time an extended characterization of stem cell markers in hWJSCs. The transcriptome profiles of hWJSCs were very different from that of the hESCs and hECCs and do not share the upregulated expression of known pluripotency markers (POU5F1, SOX2, NANOG and LIN28) required for hESC renewal and induction of pluripotency. Despite this difference, hWJSCs are multipotent and are able to differentiate into a wide variety of tissues [16, 19–22, 24]. Unlike hESCs and hECCs, hWJSCs do not produce teratomas in immunodeficient mice [16] and when injected into non-immunosuppressed rat brains did not induce any tumours at 6 and 12 weeks [23]. hWJSC-derived tissues also do not appear to induce teratomas [22]. The absence or very low expression level of key pluripotency markers such as POU5F1, NANOG, LIN28 and SOX2 may explain why hWJSCs do not produce teratomas when transplanted as undifferentiated cells [16] or as hWJSC-derived tissues [22] and is also supported by studies that have identified the ES-like signature in cancers [48, 49].

Several studies have reported the detection of pluripotency stem cell markers (POU5F1, SOX2, NANOG) in

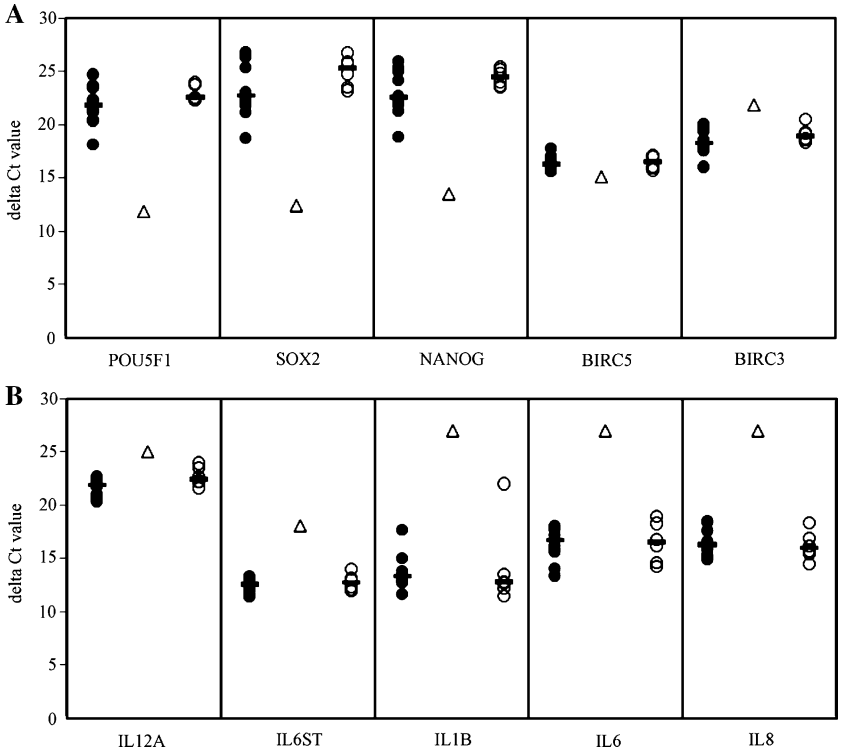


Fig. 4 Validation of selected genes by Real-time PCR. **a** The expression of the pluripotency markers (POU5F1, SOX2, NANOG) and anti-apoptosis genes (BIRC3, BIRC5), and **b** selected cytokines in hWJSCs were determined and compared to hESCs. The Δ*Ct* values were normalized to 18S rRNA. The expression level is inversely proportional to the the Δ*Ct* value and Δ*Ct* value≥27 was considered as undetermined. Pluripotency markers were detected in hWJSCs at low levels using the qRT-PCR assay. hESCs [▵], early-passage hWJSCs [●] and late-passage hWJSCs [○]

human and porcine WJSCs using qRT-PCR [16, 37, 40, 46, 47]. However, our qRT-PCR and microarray data performed on a fairly large panel of early and late passage hWJSCs confirmed that pluripotency markers were expressed at very low levels compared to hESCs. In the human there are 2 isoforms of POU5F1 (OCT4A and OCT4B) and therefore the detection of POU5F1 is potentially problematic because only OCT4A is involved in the maintenance of pluripotency while OCT4B is expressed ubiquitously [50, 51] and furthermore POU5F1 has multiple pseudogenes [52]. Therefore, caution should be taken in the design and interpretation of expression and immunological data for POU5F1 as several false positive reports have occurred because of non-specificity of the probes, PCR primers and antibodies used [53]. Both immunostaining and FACS results from our previous work [16] together with results from our ongoing work, rule out the possibility of the existence of small sub-populations of hWJSCs expressing pluripotency-associated markers in higher levels compared to the general population of hWJSCs.

Specific levels of POU5F1 are required for the maintenance of pluripotency in mouse embryonic stem cells (mESCs) and a modest 50% decrease leads to differentiation to trophectoderm while an increase leads to primitive endoderm and mesoderm [54]. Interestingly, POU5F1, SOX2, and NANOG have been reported to be expressed in various types of mouse MSCs [55]. The requirement for POU5F1 in the self-renewal and maintenance of adult mouse MSCs was subsequently investigated using a knockout mice model [56]. The targeted knockout of POU5F1 in the adult mouse MSCs did not impair their derivation and normal functional capabilities. This suggests that low levels of POU5F1 are insufficient to play a major role in the maintenance of MSC multipotency. In contrast, the knockdown of POU5F1 in hBMMSCs causes them to exit from the cell cycle and a subsequent decrease in cell growth rate [57]. The study also showed that POU5F1 bound to similar sites in hESCs and hBMMSCs, as revealed by chromatin immunoprecitation. However, the forced expression of POU5F1 and NANOG in hBMMSCs is reported to improve their stemness properties [58]. It is likely that because of their very low expression levels in hWJSCs, these pluripotency-associated markers (in particular POU5F1) may not play any important functional roles or there may be differences in the requirement of POU5F1 in the different types of MSCs. Further RNAi knockout studies of the pluripotency markers may be required to clarify how POU5F1 with its pluralistic roles actually functions in fetal and adult stem cells.

Since hWJSCs originate from the embryonic epiblast and lie between hESCs and adult MSCs on the developmental map [14, 17] they may be considered more primitive than adult hBMMSCs [13]. Indeed, the results of the present study showed that hWJSCs have a higher number of stem cell and germ lineage markers compared to other fetal and adult MSCs which is consistent with other published studies [36, 40]. hWJSCs also share some

properties unique to fetal-derived MSCs such as faster proliferation rates and greater *in vitro* expansion capabilities compared to adult hBMMSCs [22, 59]. Also, stemness properties in hWJSCs remained for much longer duration (up to 10 passages) compared to hBMMSCs where stemness usually lasts for only up to about 3 passages [13]. Our DNA microarray analysis clearly shows that hWJSCs retained a large panel of markers for all three primordial germ layers regardless of passage length and this would substantiate their wide multipotency. In fact they have been successfully differentiated into a variety of lineages including bone, pancreatic islets, neurons, and cartilage [19–24]. It also appears that MSCs from different tissue sources exhibit different and unique gene expression profiles. The results of the present study showed that hWJSCs maintain their unique multipotent characteristics for longer periods *in vitro* compared to hBMMSCs and as such they may be a better substitute to the more popularly used hBMMSCs for transplantation therapy.

hWJSCs Have Immunomodulatory Properties

MSCs in general have been regarded as hypoimmunogenic cells [22, 60, 61] and it is postulated that different tissue sources of MSCs may have different cytokine expression profiles [62]. hWJSCs are non-immunogenic as they do not elicit an immune response [22, 63] and they also express a wide range of cytokines [60, 64, 65]. Our transcriptome analysis provides further evidence that the ability of hWJSCs to modulate immune responses is due to the upregulation of genes associated with immune system processes related to antigen processing and presentation, immune system development and activation. In comparison to the results of Yoo et al. [65], we detected more cytokines at higher expression levels in our hWJSCs. For example, our DNA microarray analysis indicated high expression of IL1A, IL1B, and VEGF transcripts in hWJSCs, but Yoo et al [65] did not detect these specific cytokines when supernatants collected from hWJSC cultures were examined using a multiplex ELISA-based assay. They however confirmed the expression of IL-12 (p40), IL6, and IL8 in hWJSCs. The expression of IL6 together with VEGF has been previously shown to be important in the immunosuppressive properties of MSCs [66]. The combination of HLA Class I (+) and HLA Class II (−) expression in hWJSCs is also essential for immunosuppressive effects at the fetal-maternal interface [67, 68].

In addition, hWJSCs express high levels of the MHC Class I molecule, HLA-G, which is mainly expressed by trophoblast and placental cells and is important for immune tolerance during implantation by inhibiting the decidual natural killer cells [37, 61, 69, 70]. CD200 (OX-2) has been reported to exert an immunosuppressive effect and to prevent fetal rejection in pregnancies [71, 72]. CD200 has also been shown to play an important role in immune tolerance of allogeneic skin and cardiac transplantation in the mouse model [73]. Thus, hWJSCs may be useful for allogeneic transplantation as either hWJSCs *per se* or hWJSC-derived tissues without major issues of immune rejection. It is currently unknown if hWJSC-derived tissues would be able to maintain expression of the immune tolerance molecules and HLA signature after differentiation and transplantation in vivo.

The Anticancer Effects of hWJSCs may be Mediated Through Induction of Apoptosis or Immune Cell Recruitment

Our microarray analysis revealed that hWJSCs had an upregulation of biological processes involved in the induction of apoptosis and immune system regulation. hWJSCs have been previously shown to be able to seek out and kill cancer cells [25, 26]. Although the tumour attraction mechanisms for hWJSCs are still unclear, it does appear however that the expression of cytokines and chemokines may be responsible as is the case for other hMSCs [74]. Furthermore, hWJSC-conditioned medium was able to kill human hepatocarcinoma cells (HepG2) (unpublished data) within an *in vitro* teratoma system [75]. This indirectly implies that one possible mechanism for the anticancer effects of hWJSCs may be via the secretion of specific factors or signalling molecules that trigger a killing pathway without going through the immune system processes. However, IL8, IL1B, and IL6 which are highly expressed in hWJSCs act as mediators in the inflammatory response [76] and the secretion of IL8 by hWJSCs at the tumour site may attract immune cells to kill the cancer cells. IL12A and IL12B, respectively code for the p35 and p40 subunit of the IL12 heterodimer [77, 78] and IL12 has been shown to be a potent anti-tumour agent [79, 80]. An appropriate administration method for hWJSCs or its conditioned medium to induce a specific anti-cancer action without causing too much harm to the patient normal cells may thus have to be carefully worked out before successful clinical application is possible.

The mechanisms responsible for the homing and anticancer behaviour of hWJSCs have not yet been properly identified. One interesting finding is that SEMA3C (also known as Semaphorin E) was found to be highly upregulated in hWJSCs. Since SEMA3C promotes cell migration [81, 82], and is a high affinity ligand for neuropilin/ plexin receptors that are over-expressed by angiogenic endothelial cells and many tumour cells [83], this may be a possible mechanism in which hWJSCs home into tumour cells. Our transcriptome analysis however suggests activation of the apoptosis pathway is likely to

be important, although it is unclear how this translates into the anticancer effects observed so far. Transcriptome profiling of hWJSCs co-cultured with cancer cells will throw light on the expression pattern of genes that are regulated during hWJSC-induced cancer apoptosis.

hWJSCs may Lack the Propensity to Become Neoplastic

BIRC5 is highly expressed in human cancer cell lines and prevents cell death by apoptosis through inhibition of caspase activity [84]. Additionally, BIRC5 is required for teratoma formation induced by hESC in SCID mice as a survival factor [43]. hWJSCs do not show cancer-supportiveness nor the potential to turn cancerous as is the case for hESCs which acquire malignancy after long term culture [85]. Our previous studies have shown that there were neither chromosome abnormalities nor aberrant behaviour in hWJSCs at 50 passages [16]. Our DNA microarray results confirm that hWJSCs express different and higher numbers of tumour suppressor genes and oncogenes compared to hESCs and hECCs and additionally BIRC5 expression was lower in hWJSCs. It appears that together with the low expression levels of POU5F1, SOX2, and NANOG that have been implicated as cancer signatures [47, 48] hWJSCs may have a diminished propensity to become neoplastic.

All the hWJSC lines in this study were grown in the same culture media formulations and under similar culture conditions. It would not be unusual to see some differences in transcriptomes between the hWJSCs and the other stem cell types as the different culture media formulations in which the different stem cell types were grown may affect the cells differently, but the major differences in transcriptomes between the various stem cell types would continue to exist. In this study, it was important to first establish the transcriptome profile of hWJSCs and secondly examine whether there were significant differences between the stem cell types.

Our microarray analysis revealed minor differences in gene expression between early and late passages of hWJSCs, with approximately 108 genes identified (at 2-fold differences). However, these differences were not noted in all the pairwise comparisons and are therefore likely to be due to culture conditions, etc. In their proteomic study, Angelucci et al [86] reported senescence setting in very rapidly. We did not observe similar effects and have instead presented data that hWJSCs can be cultured robustly with minimal changes as judged by their gene expression profiles. Since the preparation of hWJSCs and culture differences are different in the study by Angelucci et al [86] it is unclear where the differences lie.

In summary, hWJSCs are a valuable source of fetal stem cells that are ethically non-controversial, can be produced in abundance, have high proliferation rates, are widely multipotent, do not produce teratomas or other tumours and are hypo-immunogenic. These desirable characteristics together with its homing and anticancer behaviour make it a novel stem cell choice for future cell based therapies and of therapeutic value for the treatment of certain cancers.

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