

Article

Comparative growth behaviour and characterization of stem cells from human Wharton's jelly



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Abstract

Human embryonic stem cells (hESC) face ethical sensitivities and the problem of teratoma formation. Although Wharton's jelly stem cells (WJSC), also of embryonic origin, may not face such ethical concerns, it is not definitely known whether under hESC culture conditions they would be as pluripotent as hESC. WJSC grown on plastic showed two types of morphology (epithelioid and short fibroblastic) in primary culture depending on the culture medium used, and only fibroblastic morphology when passaged. When grown in the presence of hESC medium on mouse feeder cells, they produced atypical colonies containing hESC-like cells with high-nuclear cytoplasmic ratios and prominent nucleoli. They were positive for the hESC markers Tra-1-60, Tra-1-81, SSEA-1, SSEA-4, Oct-4 and alkaline phosphatase, negative for SSEA-3, showed normal karyotypes, developed embryoid body (EB)-like structures, did not produce teratomas in SCID mice and differentiated into neuronal derivatives. They were also positive for the mesenchymal CD markers (CD105, CD90, CD44), negative for CD34 and HLA, and although nine out of 10 embryonic stem cell genomic markers were detectable, these were expressed at low levels. WJSC are thus not as pluripotent as hESC but widely multipotent, and have the advantages of being able to be scaled up easily and not inducing teratomas.

Keywords: characterization, embryonic stem cells, morphology, Wharton's jelly stem cells

Introduction

Human stem cells derived and propagated from embryonic or fetal tissues such as preimplantation embryos, germ cells, fetal organs and the umbilical cord have tremendous potential in the preparation of tissues for transplantation therapy. However, stem cells derived from embryos and abortuses face sensitive ethical issues in their large scale production for clinical application, while those from the umbilical cord face less controversial issues, since this anatomical structure is usually discarded at birth.

The human umbilical cord contains two arteries and a vein and a mucilaginous jelly-like substance (Wharton's jelly) that lies between the blood vessels that helps to prevent strangulation of the vessels (Chacko and Reynolds, 1954). Stem cells have been reported in umbilical cord blood, the Wharton's jelly, and in

other perivascular mesenchymal areas within the cord (Kadner *et al.*, 2002; Mitchell *et al.*, 2003; Wang *et al.*, 2004; Sarugaser *et al.*, 2005). Two types of stem cells that have been identified in umbilical cord blood are haemopoietic stem cells (HSC), which are blood-forming, and mesenchymal stem cells (MSC), which are supposedly pluripotent (Kogler *et al.*, 2004; Lee *et al.*, 2004; Weiss *et al.*, 2006). Several potential clinical applications using stem cells derived from the human umbilical cord blood have been reviewed by Ghen *et al.* (2006). Stem cells within the umbilical cord other than those in cord blood have been broadly termed umbilical cord matrix stem cells (UCMSC). Whether the stem cells within the Wharton's jelly *per se* (WJSC) and those in the perivascular mesenchymal areas are all one and the same or have different behavioural and characterization properties is

not known. In addition, the isolation methods for these various stem cell sources within the umbilical cord appear to be very different between various reports (Mitchell *et al.*, 2003; Wang *et al.*, 2004; Sarugaser *et al.*, 2005). Some workers scrape off the Wharton's jelly to isolate stem cells from the scrapings, keeping the blood vessels intact (Romanov *et al.*, 2003), while others first remove the blood vessels and then scrape off the Wharton's jelly with a scalpel (Wang *et al.*, 2004), or immerse the entire remaining part of the cord in enzyme solutions to separate stem cells from the perivascular mesenchyme and the Wharton's jelly (Mitchell *et al.*, 2004; Weiss *et al.*, 2006). Other reports remove the blood vessels together with surrounding Wharton's jelly, tie up the ends of each vessel into a loop, which is then immersed in enzyme solutions to separate stem cells from Wharton's jelly and perivascular mesenchyme (Sarugaser *et al.*, 2005). In another report, the blood vessels were first removed and the remaining tissue chopped with scissors and scalpels into pieces, which were immersed in an enzymatic solution to digest individual cells for culture (Karahuseyinoglu *et al.*, 2007). It is therefore important that proper foolproof isolation methods be identified to make reliable investigations as to the exact source and nature of stem cells within the human umbilical cord. Claims have also been made that UCMSC have wide pluripotential properties based on characterization tests used for mesenchymal stem cells such as the CD and HLA markers, molecular genetic markers and in-vitro differentiation into neurons, cardiomyocytes, bone, chondrocytes and adipose tissue (Wang *et al.*, 2004). The various components of the umbilical cord are of embryonic origin and the characterization tests for human embryonic stem cells (hESC) such as the surface marker antigens (SSEA, Tra series), Oct 4, molecular markers for ectoderm, mesoderm and endoderm, alkaline phosphatase, karyotype, severe combined immunodeficient (SCID) mice teratoma formation, telomerase, colony formation and in-vitro differentiation, have not been comparatively carried out between UCMSC and hESC to establish whether UCMSC are genuinely pluripotent, like hESC, or are multipotent, like mesenchymal stem cells. Furthermore, all reports thus far describe derivation and propagation of UCMSC on plastic without evaluating the use of feeder cells and culture media ingredients similar to hESC and as such the behaviour of UCMSC on feeder cells in the presence of hESC media is not known. The results of such studies will project the true value of UCMSC and its possible substitution for the ethically controversial hESC. To ensure a homogeneous and specific stem cell population, a protocol was used to isolate stem cells only from one specific area of the umbilical cord, i.e. the Wharton's jelly. For this specific reason, the cells isolated in this study are referred to as Wharton's jelly stem cells (WJSC).

This report describes in detail: (i) the morphological growth characteristics *in vitro* of primary and passaged WJSC grown separately on plastic and on inactivated mouse embryonic fibroblast feeder (MEF) cells in culture media conventionally used to propagate UCMSC (Weiss *et al.*, 2006) and hESC (Richards *et al.*, 2002) and (ii) the detailed characterization of WJSC using a battery of hESC and MSC markers.

Materials and methods

Cell culture

Informed patient consent and Institutional Review Board (DSRB, National Healthcare Group) approval was obtained for

collection of umbilical cords from human subjects for this study (D/06/059).

The protocol for isolation of WJSC described here are different from published reports. Umbilical cords (approximately 20 cm) were collected from seven patients at full term delivery in transport medium (Hank's Balanced Salt Solution supplemented with antimycotic-antibiotic solution, Invitrogen Life Technologies, CA, USA), stored at 4°C and processed within 12 h after collection.

Each cord was cut into 2 cm parts, washed well in fresh transport medium to remove blood and debris, and gently teased to squeeze out any trapped blood within the veins and arteries. Each 2 cm piece was cut open lengthwise with sterile scissors and placed with its inner surface facing down in a 60 mm Petri dish containing an enzymatic solution. The enzymatic solution comprised of 2 mg/ml collagenase I, 2 mg/ml collagenase type IV and 100 IU/ml of hyaluronidase (Sigma, MO, USA) in Dulbecco's modified Eagle's medium (DMEM) (high glucose) (Invitrogen). The volume of the enzymatic solution in the Petri dish was such that it only allowed the Wharton's jelly to come into contact with the enzymes. Approximately, five to six such cord pieces were placed in each Petri dish and incubated at 37°C for 45 min. The purpose of the exposure of only the inner surface of the cord pieces to the above concentrations of enzymes for a specific duration was to loosen up the WJSC.

After enzymatic digestion, cord pieces were transferred separately to fresh equilibrated DMEM medium, face up this time. Using the curved surface of watchmaker forceps, the Wharton's jelly surrounding the umbilical cord blood vessels was gently detached into the DMEM medium and the remaining cord pieces containing the vessels were discarded. Care was taken as much as possible to gently detach (without scraping) only the Wharton's jelly into the DMEM medium, leaving the blood vessels intact within the cord. The DMEM medium containing the Wharton's jelly was transferred to 14 ml conical tubes and resuspended with sterile Pasteur pipettes to break up the gelatinous masses. The suspension was then centrifuged at 300 *g* for 5 min, supernatant discarded and cell pellet resuspended in fresh DMEM medium and washed again. The final pellet was resuspended equally into either UCMSC medium comprising fresh DMEM (high glucose) medium supplemented with 15% fetal bovine serum (FBS), glutamine and antibiotic-antimycotic solution (Invitrogen), or hESC medium containing DMEM (high glucose), 20% FBS, glutamine, insulin-transferrin-selenium (ITS), bovine fibroblast growth factor (bFGF) and antimycotic-antibiotic solution (Invitrogen).

The WJSC in UCMSC or hESC medium were seeded separately into small Petri dishes coated with mitomycin C (MMC) (Sigma) treated MEF (Invitrogen) and on plastic in the absence of MEFs. All four types of dishes were incubated at 37°C in a 5% CO₂ in air atmosphere. Separately, hESC [HES3 and HES4 lines; ES Cell International Pte Ltd, (ESI), Singapore] were grown in hESC medium on MMC inactivated MEF at the same passages and under the same conditions as the WJSC to act as controls (Richards *et al.*, 2002). Cell growth was monitored daily with changes of medium every 48 h for all dishes (WJSC and hESC).

When the WJSC became confluent on plastic or formed a reasonable number of colonies on MEF in primary culture, they were passaged by detachment with trypsin or dispase, washed, and re-seeded into new plastic dishes coated or uncoated with MEF in the presence of DMEM or hESC medium. The same was done for the hESC. The WJSC could be successfully frozen in liquid nitrogen, thawed and expanded in large numbers.

Embryoid body (EB) formation

hESC colonies were passaged enzymatically with collagenase IV (Invitrogen) by the transfer of small clumps of undifferentiated HES-3 and HES-4 cells to fresh MEF feeders at 7- to 8-day intervals. HES-3 and HES-4 EBs were obtained by culturing clumps of 50–100 cells for 24 h in non-stick 6-well tissue culture dishes (Nunc, Roskilde, Denmark). Cell culture medium used to initiate EB formation consisted of 90% DMEM, 10% serum replacement (Invitrogen), 1× antimycotics and 2 mmol/l L-glutamine (Invitrogen). Serum replacement was used in place of FCS for EB formation to prevent hESC clumps from adhering to the culture dishes. The same protocol was used to produce EB from the WJSC.

Surface marker antigens

For immunofluorescence demonstration of the stem cell markers Tra-1–60 and Tra-1–81, WJSC and hESC were fixed with 100% ethanol, and for SSEA-1, 3 and 4, the cells were fixed with 90% acetone in water. The monoclonal antibodies for the detection of the markers were from Development Studies Hybridoma Bank (Iowa City, IA, USA). Antibody localization was carried out using anti-mouse immunoglobulin conjugated to fluorescein isothiocyanate (Sigma).

CD markers

Standard flow cytometry and immunofluorescence analysis was carried out at early and late passages for WJSC and hESC for a battery of CD markers.

Karyotype

Giemsa banded karyotypes were prepared using the conventional method of treatment with colcemid (Sigma) to arrest mitosis at metaphase followed by hypotonic treatment of the cells with hydrochloric acid (HCl), and fixation with 3:1 methanol:glacial acetic acid. Metaphases were trypsin banded and stained with Giemsa. A total of 20 metaphases per sample were examined.

Alkaline phosphatase

Alkaline phosphatase activity was detected with the Vector Red Alkaline Phosphatase Substrate Kit I (Vector Labs, Burlingame, CA, USA) and viewed with rhodamine excitation and emission filters.

SCID mice

Approximately 5×10^6 WJSC were injected with a sterile 25G needle into the thigh muscle of SCID mice. The 24 thighs

(right and left) from 12 mice were injected with cells from three donor cords for each arm (WJSC grown on plastic with UCMSC medium, WJSC on MEF with UCMSC medium, WJSC on plastic with hESC medium and WJSC on MEFS with hESC medium). Similar injections were carried out for six mice for the two hESC lines (controls). The mice were killed at 16 weeks and thighs dissected carefully to examine for any tumours. Any tumour tissue that was present was dissected out, fixed in 4% (v/v) formaldehyde, embedded in paraffin and examined histologically after haematoxylin and eosin staining.

Quantitative real-time polymerase chain reaction (PCR)

Total RNA was isolated using Trizol™ reagent (Gibco BRL, USA), and RNA quality and quantity was measured with a NanoDrop™ spectrophotometer (NanoDrop Technologies, CA, USA). All samples were treated with DNase I prior to first strand synthesis. First strand cDNA synthesis was carried out with random hexamers using the SuperScript™ first strand synthesis system (Invitrogen) according to the manufacturer's protocol. Primer sequences were either taken from published studies (summarized in **Table 1**) or designed using the Primer Express Software v3.0 (Applied Biosystems, USA). Primer concentrations were optimized prior to the conduct of the experiment. Quantitative real-time (qRT) PCR analysis was performed with the ABI PRISM 7500 Fast Real-Time PCR System (Applied Biosystems) using SYBR green. Each reaction was performed in quadruplicate with first-strand cDNA equivalent to 20 ng input total RNA. Equal amounts of cDNA were used for all PCR reactions. After an initial denaturation for 10 min at 95°C, PCR was carried out for 40 cycles (95°C for 15 s, 60°C for 1 min). Actin-β (ACTB), glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and TATA-binding protein (TBP) were used as the normalization controls; untreated samples were used as the calibrator. Relative quantification was performed using the comparative CT ($2^{-\Delta\Delta CT}$) method.

Neuronal differentiation

Differentiation of WJSC into neuronal cells was induced using the method of Woodbury *et al.* (2000). The WJSC were first incubated overnight with bFGF (10 ng/ml) in DMEM supplemented with 20% FBS. The cells were then exposed to 2% dimethylsulphoxide (DMSO) and butylated hydroxyanisole (BHA) (200 μmol/l) in DMEM supplemented with 2% FBS. After 5–6 h, the medium was changed to a solution of potassium chloride (KCl) (25 mmol/l), valproic acid (2 mmol/l), forskolin (10 μmol/l), hydrocortisone (1 μmol/l) and insulin (5 μg/ml) (Sigma) for long-term induction and maintenance of neuronal differentiation. Quantitative real-time PCR was then carried out on the treated cells.

Results

Morphological characteristics

Using the isolation method described in the present study, the WJSC of all seven patients were successfully propagated in

Table 1. Primer sequences for CD markers used in the analysis of Wharton's jelly and human embryonic stem cells.

Target	Forward primer (5'→3')	Reverse primer (5'→3')
ABCG2	GATTTACGGCTTTGCAGCATAA	AAGGATTGTTTCCTGTTGCATTG
ACTB	TGCCGACAGGATGCAGAAG	CTCAGGAGGAGCAATGATCTTGAT
AFP	GAGGGAGCGGCTGACATTATT	ACCAGGGTTTACTGGAGTCATTTCC
Beta-III-tubulin	GGCCAAGTTCTGGGAAGTCA	CGAGTCGCCCCACGTAGTTG
Brachyury	TTCTCCAACCTATTCTGACAACCTCA	ATTCCAAGGCTGGACCAATTG
CXCR4	GGCCTGAGTGCTCCAGTAG	TGTATATACTGATCCCCTCCATGGT
DNMT3B	ACACCCAGTCATGCCAAA	AGTTCGGACAGCTGGGCTTT
GDF3	ACCAAGGTTTCTTTTCTTTACCCAAA	TTGATGGCAGACAGGTTAAAGTAGA
GFAP	GCGAGGAGAACCGGATCAC	GTGGCCTTCTGACACAGACTTG
IPF1	GCTGGCTGTCATGTTGAACCTTG	GGCGGTTTTGGAACCAGAT
ISL1	TCGCCTTGACAGAGTGACATAGA	AGAGCCCGTCCCTCTTCT
Nanog	AGAACTCTCCAACATCCTGAACCT	TTCTGCCACCTCTTAGATTTTCATTC
Nestin	AGCCCTGACCACTCCAGTTTAG	CCCTCTATGGCTGTTTCTTTCTCT
POU5F1	GGAGGAAGCTGACAACAATGAAA	GGCCTGCACGAGGGTTT
REX1	GCCTTCACTCTAGTAGTGTCTACAGT	GGCAGTAGTGATCTGAGTAAGCTGTCT
SOX2	ACAGCAAATGACAGCTGCAAA	TCCGCATCGCGGTTTTT
TERT	CATTTTTCTGCGCGTCAT	GCGACATCCCTGCGTTCT
Vimentin	TCCAAACTTTTCTCCCTGAAC	GGGTATCAACCAGAGGGAGTGA

primary culture and in serial culture on plastic and MEF for up to 50–55 passages.

Primary culture

The isolation method used in the present study consistently produced two types of cell morphology in primary culture for all patient samples depending on the culture medium used, and whether the cells were grown on plastic or feeder cells. When WJSC were grown on plastic in the presence of hESC medium, the cells produced an epithelioid morphology (**Figure 1A, B**) and when grown on plastic in the presence of UCMSC medium they produced short fibroblast-like cells (**Figure 1D, E**). Both the epithelioid and short fibroblast-like cells had high nuclear–cytoplasmic ratios (**Figure 1**).

When the WJSC from the same patients were plated on MMC-treated MEF in the presence of UCMSC medium, the short fibroblast-like morphology remained the same as that seen on plastic, with no formation of colonies as typically seen with hESC.

However, when the WJSC were grown on MMC treated MEF in the presence of hESC medium they displayed colonies in primary culture containing small circular cells with high nuclear–cytoplasmic ratios similar but not exactly typical to colonies observed with hESC on MMC-treated MEF in hESC medium (**Figure 2**).

On plastic in the presence of UCMSC or hESC medium, WJSC attached in 3–4 days after seeding, and confluence was achieved in 10–14 days when grown in small plastic Petri dishes (changing of medium every 48 h).

Passages

When the WJSC were passaged, the cells with epithelioid or short slender fibroblast-like morphology seen in primary culture on plastic, transformed completely to long slender fibroblast-like cells from the first passage onwards irrespective of the culture medium used (**Figure 1C, F**). At late passages (50–55), cell growth on plastic slowed down and the cells took on a wide banded appearance which, when confluent, showed piling up of cells in certain areas that resembled little cell mounds. These mound-like structures did not exceed more than 0.4 mm in diameter.

Serial passaging of the WJSC on MEF in hESC medium resulted in the maintenance of colonies containing small circular cells with high nuclear–cytoplasmic ratios.

Embryoid body formation

When the embryoid body induction protocol for hESC was used on the WJSC, atypical embryoid body-like structures were produced (**Figure 3A**). These appeared as small circular spheres that contained within them cells that underwent degeneration very fast in culture and turned dark brown (**Figure 3B**). When histological sections of these spheres were examined, no cell derivatives of the three primordial germ layers were observed.

Karyotype

Giemsa-banded karyotypes showed stable normal chromosome complements at P1, P5, P10 and P50 passages in WJSC grown both on plastic as well as MEF in the presence of either UCMSC or hESC culture media.

Alkaline phosphatase

Immunohistochemistry evaluation of WJSC showed primary and passaged cells (epithelioid and fibroblast) from all patient isolations to be positive for alkaline phosphatase activity compared with hESC. In order to confirm this, RT-PCR evaluation of the same isolations also showed positive alkaline phosphatase activity.

hESC surface marker antigens

The WJSC of all the three patient isolations (grown on plastic and MEF) that were analysed stained positive for Tra-1-60, Tra-1-81, SSEA-1 and SSEA-4 and were negative for SSEA-3, while the two hESC lines (HES3 and HES4) were positive for all the above markers, including SSEA-3 (**Table 2**).

CD markers

Flow cytometric plots showed positive expression in WJSC for CD105, CD90 and CD44 markers in all six patients analysed, while expression of CD45, CD34, HLA-DR, DP, DQ-/CD30-, HLA-DR, DP, DQ+/CD30+, HLA-DR, DP, DQ+/CD30- and HLA-DR, DP, DQ-/CD30+ markers was negative. The hESC were negative for HLA-DR, DP, DQ-/CD30+ (**Table 3**). Immunohistochemistry also confirmed the results of the above CD30 markers.

Neuronal marker differentiation

The WJSC (grown on plastic and MEF) isolated in this study showed differentiation into neuronal markers as shown in **Figure 4**. The neuronal marker genes (beta II tubulin, nestin and GFAP) showed an increase in expression when neuronal differentiation was induced in WJSC. The data is relative to monolayer WJSC controls. Vimentin, a marker for mesenchymal stem cells, showed a decrease in expression during differentiation.

qRT-PCR analysis of markers of pluripotency in WJSC

Nine out of 10 of the stem cell marker genes that were assayed were detectable in all four WJSC samples analysed. However, these stem cell marker genes were expressed at low levels in WJSC as compared with hESC (**Figure 5**). In particular, key markers of pluripotency such as POU5F1, NANOG, TERT and DNMT3B were found to be highly down-regulated in WJSC (**Figure 5**). IPF1 expression was absent in three patients but detectable only in one patient sample (**Figure 5**). Interestingly, vimentin expression was much higher in all four WJSC samples as compared with the hESC sample. AFP and nestin were also expressed at much lower levels in WJSC, while TUJ1 expression was found to be higher in two WJSC samples. Three endogenous controls, ACTB, GAPDH and TBP, were used for qRT-PCR analysis, and data displayed in **Figure 5** are representative of gene expression normalized to

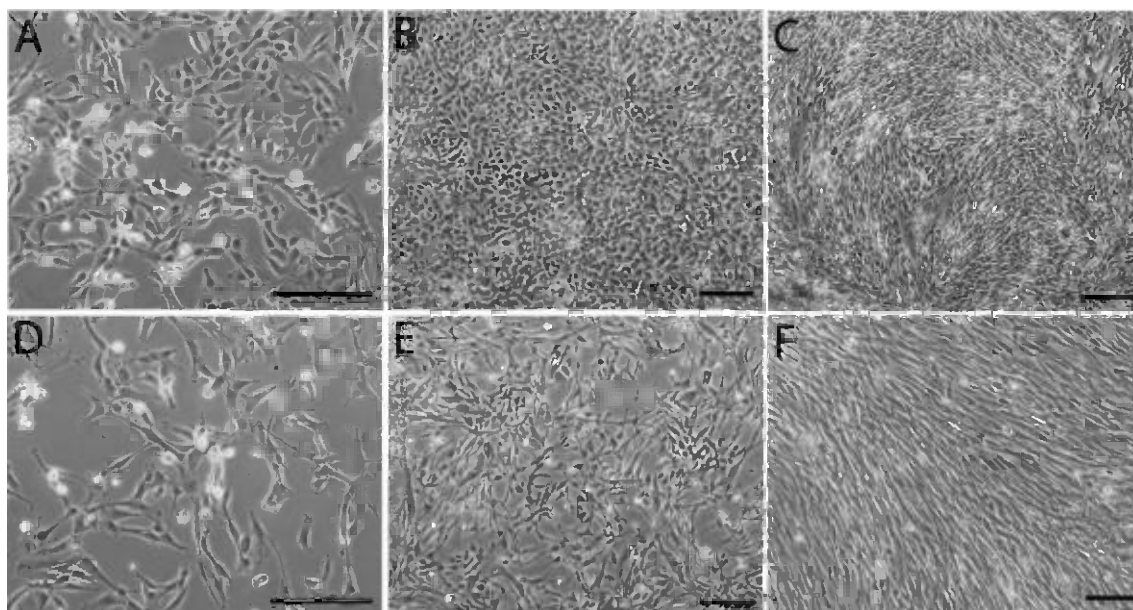


Figure 1. Morphological behaviour of Wharton's jelly stem cells (WJSC) grown on plastic *in vitro* in the presence of umbilical cord matrix stem cell (UCMSC) or human embryonic stem cell (hESC) medium. (A) Day 3 primary culture of WJSC on plastic in hESC medium growing as epithelioid islands. (B) Day 12 confluent primary culture of WJSC on plastic in hESC medium showing epithelioid morphology being maintained. (C) First passage WJSC in hESC medium showing epithelioid cells transformed to confluent fibroblast-like cells morphology. (D) Day 3 WJSC on plastic in UCMSC medium showing short fibroblast cells. (E) Day 8 primary culture of WJSC on plastic in UCMSC medium showing short to long fibroblasts. (F) First passage WJSC on plastic in UCMSC medium showing cells maintaining long fibroblast morphology. Bars: (A, D) 50 μ m; (B, C, E, F) 100 μ m.

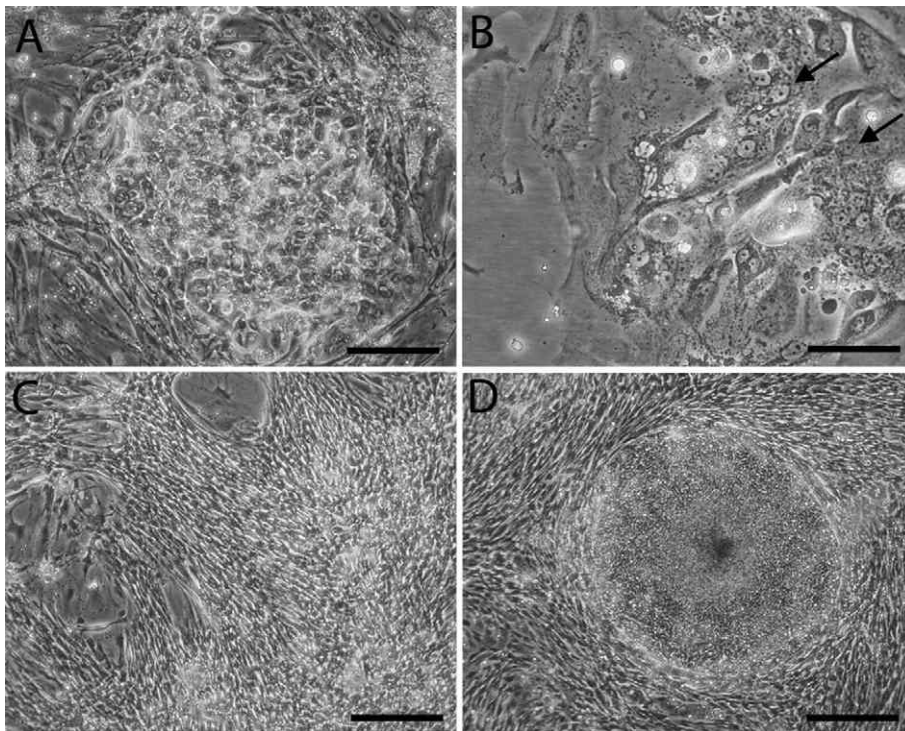


Figure 2. Morphological behaviour of Wharton's jelly stem cells (WJSC) grown on mouse embryonic fibroblasts (MEF) in the presence of human embryonic stem cell (hESC) medium. (A) WJSC showing colony formation, which is not typically circular like hESC grown on MEF in hESC medium. (B) Higher magnification of WJSC colony grown on MEF in hESC medium showing some cells similar to hESC with large clear nuclei and prominent nucleoli (arrows). (C) WJSC colonies remaining as fibroblasts on MEF in UCMSC medium. (D) Control typical hESC colony growing on MEF in hESC medium. Bars: (A, B) 30 μm ; (C, D) 200 μm .

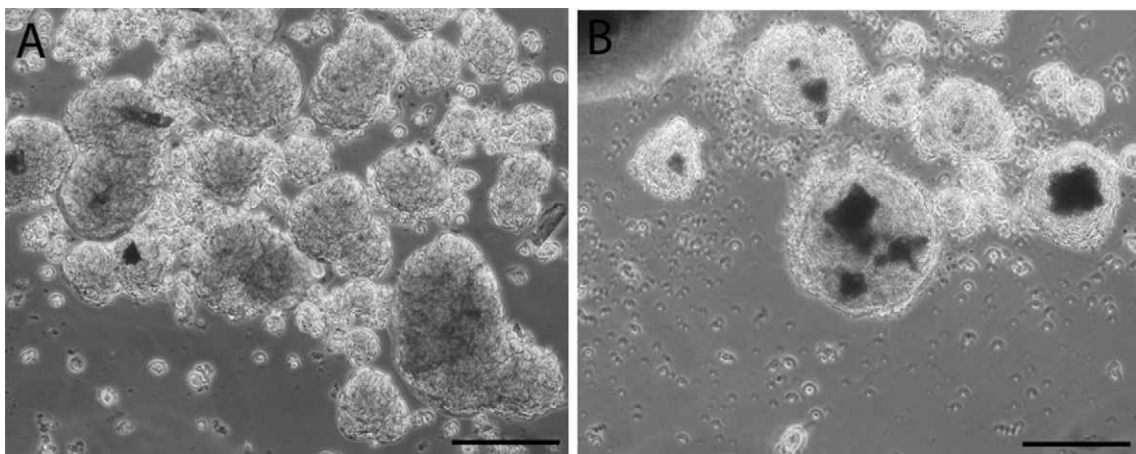


Figure 3. Embryoid body (EB) formation in Wharton's jelly stem cells. (A) EB-like structures (spheres/pseudo EB) a few days after EB initiation using human embryonic stem cell-EB protocol. (B) The same EB-like structures showing degenerating dark brown cells within them a few days later. Bars: (A, B) 300 μm

Table 2. Surface marker antigens and teratoma formation in severe combined immunodeficient (SCID) mice for Wharton's jelly stem cells (WJSC) and human embryonic stem cells (hESC).

Cell type	No. of donors	Surface marker antigens					Teratomas/SCID mice		
		Tra-1-60 ± (%)	Tra-1-81 ± (%)	SSEA-1 ± (%)	SSEA-3 ± (%)	SSEA-4 ± (%)	No. of mice	Weeks	Teratoma (n)
WJSC	3	+ (100)	+ (100)	+ (100)	- (0)	+ (100)	12 ^a	12	0
hESC	2	+ (100)	+ (100)	+ (100)	+ (100)	+ (100)	6	12	6

^aThree mice each for WJSC grown on plastic and mouse embryonic fibroblasts with hESC and umbilical cord matrix stem cell medium.

Table 3. Immunohistochemical and flow cytometric analysis in Wharton's jelly stem cells (WJSC) and human embryonic stem cells (hESC) for CD markers.

Cell type	No. of patients	Passage	CD105 ^c	CD90 ^d	CD44 ^d	CD34 ^d	HLA ^{-d} DR, DP, DQ ⁻ CD30 ⁻	HLA ^{-d} DR, DP, DQ ⁺ CD30 ⁻	HLA ^{-d} DR, DP, DQ ⁺ CD30 ⁺	HLA ^{-d} DR, DP, DQ ⁻ CD30 ⁺
WJSC	3	2-4	+	+	+	-	-	-	-	-
WJSC	3	5-10	+	+	+	-	-	-	-	-
hESC ^a	2	3	-	+	-	-	-	-	-	-
hESC ^b	2	8-10	-	+	-	-	-	-	-	-

^aCut and paste method; ^bbulk culture method; ^cimmunohistochemistry; ^dflow cytometry.

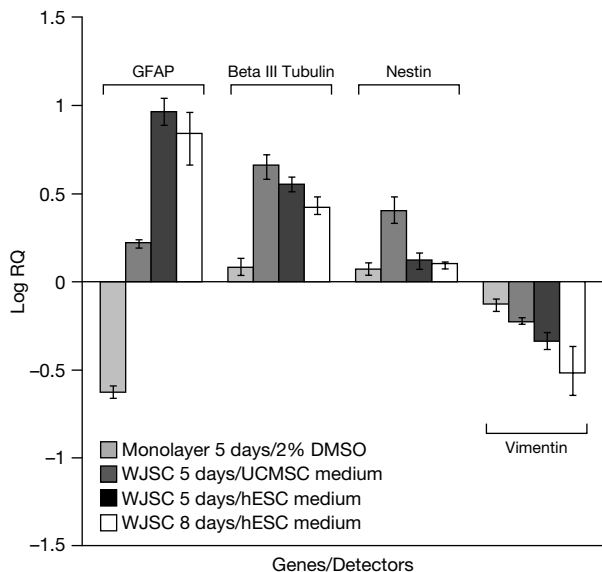


Figure 4. Quantitative real-time polymerase chain reaction gene expression of neuronal marker genes in differentiating Wharton's jelly stem cells (WJSC) relative to undifferentiated WJSC controls. hESC = human embryonic stem cell; UCMSC = umbilical cord matrix stem cell.

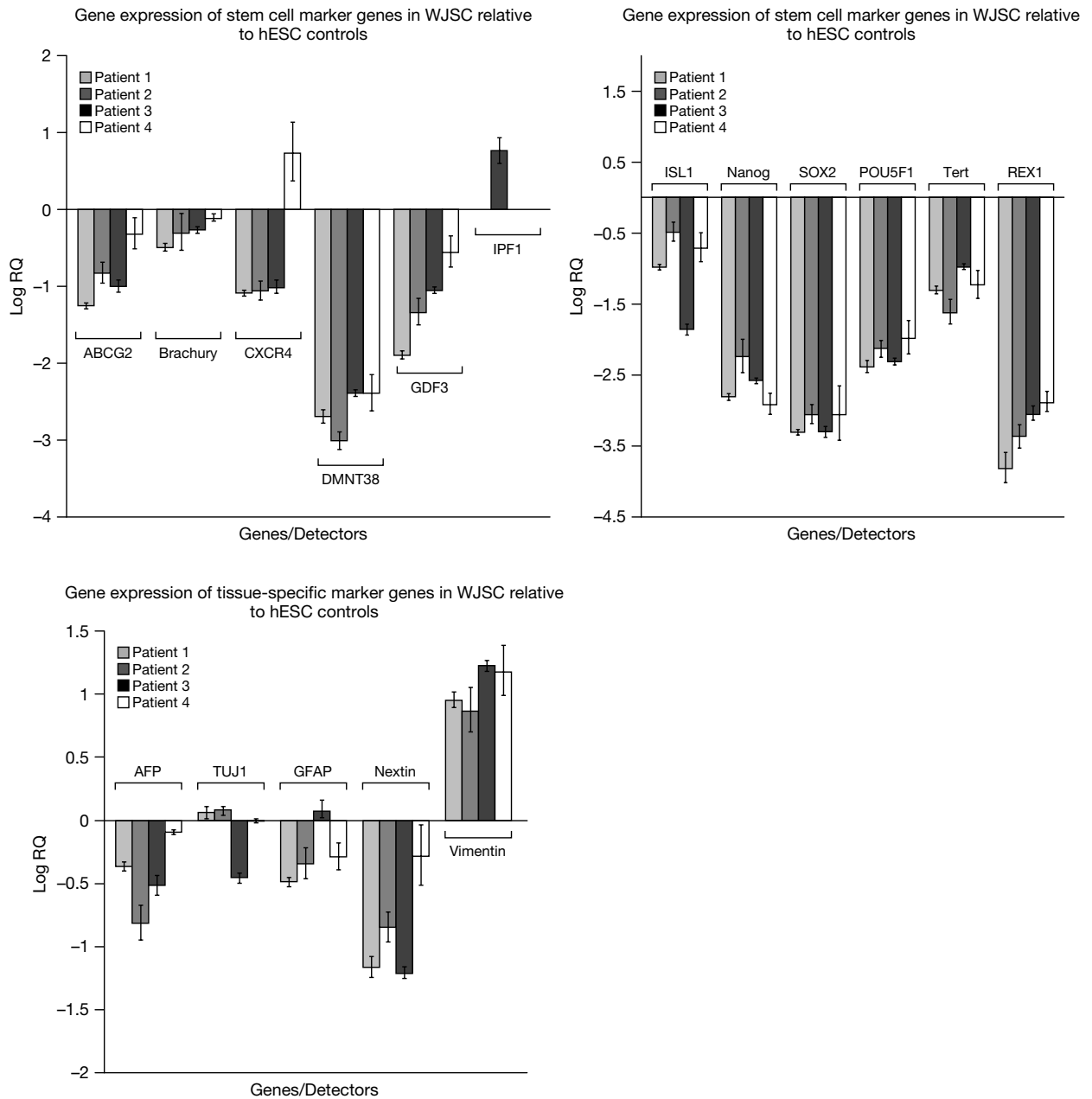


Figure 5. Quantitative real-time polymerase chain reaction gene expression of stem cell and tissue markers in Wharton's jelly stem cells (WJSC) relative to undifferentiated human embryonic stem cells (hESC). WJSC grown under hESC conditions. Actin- β endogenous control was used to normalize gene expression data. Undifferentiated HES-3 cells were used as the calibrator samples.

ACTB. Gene expression patterns were found to be consistent regardless of the endogenous control used to normalize data.

Teratoma formation in SCID mice

None of the 12 SCID mice injected with WJSC (grown on plastic or MEF) developed teratomas even after as long as 12 weeks, unlike the six SCID mice injected with hESC, where all developed teratomas (**Table 2**).

Discussion

The isolation method used in this study appeared to be efficient, simple and consistent, yielding large homogenous populations of stem cells. The gentle teasing of the Wharton's jelly under careful examination under the stereo microscope and the type of culture medium used may have been important factors in generating the pure homogeneous epithelioid cell populations seen in all cords grown in hESC medium and the pure short-fibroblastic-like populations of WJSC seen in all cords grown in UCMSC medium. Previous studies showed short or long fibroblast-like cells in primary culture in UCMSC medium, which probably originated from a mixture of cells from the Wharton's jelly, perivascular, intervascular and inner lining wall of the cord. It was postulated that the most immature cells that had greater competence to resume proliferation were located in subamniotic and intervascular regions while cells from the perivascular regions mainly comprised of highly differentiated myofibroblasts (Nanaev *et al.*, 1997).

It is well known that hESC do not attach to plastic in primary culture, and differentiate into bizarre cell types. Conventionally, hESC have required feeder monolayers such as murine or human embryonic fibroblasts for derivation and propagation (Bongso *et al.*, 2005). However, the WJSC in this study as well as those reported by other workers (Karahuseyinoglu *et al.*, 2007), are different from hESC in that they readily attach to plastic without substrates both in primary and serial culture. Usually cells of connective tissue origin grow as fibroblasts in primary culture. The fact that all WJSC primary cultures established from all isolations on plastic in the presence of hESC medium in the present study started off as epithelioid cells and remained confluent as epithelioid cells suggests that there was no contamination with connective tissue scrapings that could have detached from the perivascular and inner lining areas of the cord. Furthermore, the hESC medium appeared to have an influence on cell morphology, because the same cells grown in UCMSC medium had short-fibroblast-like morphology in primary culture.

A striking feature of the WJSC was their prolific growth and ability to expand in large numbers with serial passaging, and additionally their stemness characteristics and multipotentiality were the same whether they were grown on plastic or MEF. It thus appears that the intrinsic ability of the WJSC to attach to plastic is a unique and consistent feature, making expansion of such cells much less labour intensive and easy for scaling up in terms of numbers. The additional advantage of their growth on plastic avoids the risk of the contamination concerns from animal viruses if they are grown on MEF like hESC. The primary culture phenotypes transformed completely to fibroblast morphology with serial passaging, and such cell

differentiation *in vitro* and *in vivo* was postulated to be closely associated with changes based on intermediate filament protein remodelling (Karahuseyinoglu *et al.*, 2007).

Interestingly, cells isolated from the porcine umbilical cord Wharton's jelly (PUC) and grown on plastic showed a heterogeneous morphology of many spindle shaped cells and small round cells with high nuclear-cytoplasmic ratios in the presence of a very complex culture medium containing many ingredients over and above that used in conventional hESC medium. The small round cells often formed colonies or clusters not typical of hESC but stained positive for alkaline phosphatase (Carlin *et al.*, 2006). These observations are consistent with the human WJSC of the present study only when they were grown on MEF in hESC medium, confirming that the culture conditions and substrate may influence the morphology of umbilical cord matrix stem cells.

hESC colonies grown on feeder cells are usually uniformly positive for alkaline phosphatase. The WJSC in the present study were also positive for alkaline phosphatase irrespective of whether the cells were from confluent monolayers, colonies or from the piled up mounds. These findings are consistent with those of Carlin *et al.* (2006).

Normal karyotypes were observed for primary, early and late passages for the WJSC of the present study. This is quite contrary to most connective tissue fibroblasts grown in serial culture, which usually end up with a different transformed karyotype in late passages. Maintenance of a normal karyotype is an additional confirmed marker for stemness in the WJSC of the present study.

Interestingly, WJSC did not produce typical embryoid bodies like hESC. The cells within the EB of hESC give rise to cells of all three primordial germ layers, whereas the pseudo EB-like structures generated from WJSC were circular shining spheres that did not contain cells of the three primordial germ layers but contained cells that were undergoing degeneration, turning brown very rapidly within the spheres. This feature is consistent with the inability of WJSC to produce teratomas containing cells of all three germ layers *in vivo* in SCID mice. Similar-looking EB-like structures growing as tight adherent clusters were reported by McGuckin *et al.* (2005) from human umbilical cord blood. It thus appears that although WJSC are embryonic in origin they behave like mesenchymal stem cells (MSC) that do not have the ability to produce real EB.

The WJSC of the present study were also positive for the common CD markers, further suggesting that they have properties consistent with MSC. This is consistent with similar reports made previously that allude to the fact that cells in the Wharton's jelly should be typically classified as mesenchymal or connective tissue in origin (Karahuseyinoglu *et al.*, 2007). Since the WJSC in the present study did not express CD34 and CD45 markers but expressed CD44, CD105 and CD44, these WJSC express matrix receptors and not haemopoietic lineage receptors, suggesting similarities to MSC.

Stem cell genomic markers observed in hESC were detected in the WJSC of the present study, but at low levels. NANOG, OCT-4 and SOX-2 were shown to be expressed in PUC isolated from porcine Wharton's jelly (Carlin *et al.*, 2006). Such

transcription factors are key regulators of gene transcription in primitive stem cells, and confirm stemness in WJSC in general. These transcription factors regulate pluripotency and self renewal as seen in hESC, but since their levels were low and down-regulated in the WJSC of the present study, pluripotentiality appears to be limited in the WJSC. The high expression of vimentin, which is an intermediate filament protein characteristic of primitive neuroectoderm may be due to an early attempt at spontaneous differentiation into neurons, which is a common default pathway for stem cells grown *in vitro* (Reubinoff et al., 2000).

Interestingly, the injection of WJSC into SCID mice in numbers greater than that conventionally used for hESC did not produce teratomas even after a prolonged period of 12 weeks. This confirmed that WJSC do not possess the *in-vivo* property of producing tumours containing cells from all three primordial germ layers, a property unique to hESC and pluripotent stem cells. However, the WJSC in the present study could be differentiated into neuronal cells *in vitro* using protocols similar to human stem cells. Other reports have also shown that UCMSC can be made to differentiate into ectodermal, mesodermal and endodermal lineages *in vitro* (Kobayashi et al., 1998; Fu et al., 2004; Ma et al., 2005; Weiss et al., 2006).

The WJSC in the present study were positive for SSEA-1, which is in contrast to the results reported by McGuckin et al. (2005) on human umbilical cord blood cells. These authors stated that umbilical cord blood cells were quite restricted for stemness in the human stem cell compartment. WJSC may thus have greater stemness properties than umbilical cord blood stem cells.

The fact that the WJSC in the present study were positive for Oct 4 and certain hESC surface marker antigens (Tra-1-60, Tra-1-81, SSEA-1, SSEA-4), self renew in culture, show atypical colony formation and demonstrate differentiation into neurons, suggests that they may possess certain hESC properties, even though they were negative for SSEA-3, did not produce typical embryoid bodies and did not produce teratomas.

There are very interesting and beneficial properties of WJSC over hESC because one of the major concerns of the clinical application of hESC is the risk of renegade undifferentiated hESC producing benign teratomas *in vivo* after transplantation of terminally differentiated hESC-derived tissues.

More work needs to be carried out to confirm whether the differentiation potential reported with WJSCS or UCMSC is real, because the diverse battery of differentiation protocols used by various workers thus far on these cell types has not been exactly the same as those used for hESC. For example, WJSC or UCMSC have not been cocultured with visceral endodermal cells to produce functional cardiomyocytes (Mummery et al., 2003), nor have they been transfected with insulin producing genes to produce functional islets (Soria et al., 2001), or induced to produce neuronal cells with other agents such as *trans*-retinoic acid (Reubinoff et al., 2000). This requires very large and extensive studies, with attempts made at using a variety of differentiating strategies, the results of which warrant separate documentation.

Engraftment of human and porcine UCMSC in the brain of parkinsonian rats has been successfully demonstrated (Weiss

et al., 2003, 2006). If it turns out that WJSC are able to differentiate into functional and useful derivatives from all three germ layers, and at the same time not produce teratomas *in vivo*, WJSC will turn out to be more beneficial for transplantation therapy compared with hESC given the ethical sensitivities and tumour formation concerns of hESC.

It is quite clear from the results of the present study that WJSC cannot be concluded as truly pluripotent, but perhaps widely multipotent. If WJSC derived tissues can graft successfully with functional competence after transplantation, they would have some benefits over hESC-derived tissues as they would overcome one of the most worrying obstacles of hESC, i.e. the production of teratomas. However WJSC appear to have limited self renewal capacity and a finite lifespan in culture, because the cells from all seven patients changed their morphology to broad band-like cells and underwent degeneration by the 50th to 55th passage. In fact MSC in general can usually be propagated in culture for only 8–10 passages while retaining their phenotypic characteristics and differentiation ability (Rao, 2007). Given the fact that WJSC can be scaled up in large numbers with ease and on plastic in a short time before they lose their differentiation abilities *in vitro*, they may find good use in transplantation therapy. However, the production of reliable and functional terminally differentiated cells from WJSC, after validation in animal models will be the true test that would confirm the real benefits of WJSC over hESC.

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