

Human chorionic-plate-derived mesenchymal stem cells and Wharton's jelly-derived mesenchymal stem cells: a comparative analysis of their potential as placenta-derived stem cells

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Received: 10 June 2011 / Accepted: 11 September 2011 / Published online: 11 October 2011
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Abstract Placenta-derived stem cells (PDSCs) have gained interest as an alternative source of stem cells for regenerative medicine because of their potential for self-renewal and differentiation and their immunomodulatory properties.

This study was supported by a grant of the Korea Healthcare Technology R&D Project, Ministry for Health, Welfare & Family Affairs, Republic of Korea (A084923).

No conflicts of interest need to be declared by any of the authors.

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Although many studies have characterized various PDSCs biologically, the properties of the self-renewal and differentiation potential among PDSCs have not yet been directly compared. We consider the characterization of chorionic-plate-derived mesenchymal stem cells (CP-MSCs) and Wharton's jelly-derived mesenchymal stem cells (WJ-MSCs) among various PDSCs and the assessment of their differentiation potential to be important for future studies into the applicability and effectiveness of PDSCs in cell therapy. In the present study, the capacities for self-renewal and multipotent differentiation of CP-MSCs and WJ-MSC isolated from normal term placentas were compared. CP-MSCs and WJ-MSCs expressed mRNAs for the pluripotent stem cell markers Oct-4, Nanog, and Sox-2. Additionally, HLA-G for immunomodulatory effects was found to be expressed at both the mRNA and protein levels in both cell types. The CP-MSCs and WJ-MSCs also had the capacities to differentiate into cells of mesodermal (adipogenic and osteogenic) and endodermal (hepatogenic) lineages. Expression of adipogenesis-related genes was higher in CP-MSCs than in WJ-MSCs, whereas WJ-MSCs accumulated more mineralized matrix than CP-MSCs. The WJ-MSCs expressed more of CYP3A4 mRNA, a marker for mature hepatocytes, than CP-MSCs. Thus, we propose that CP-MSCs and WJ-MSCs are useful sources of cells for appropriate clinical applications in the treatment of various degenerative diseases.

Keywords Placenta-derived stem cells · Chorionic-plate-derived mesenchymal stem cells · Wharton's jelly-derived mesenchymal stem cells · Characterization · Differentiation potential · Human

Introduction

Mesenchymal stem cells (MSCs) have been studied as resources for cell therapy and regenerative medicine. Bone marrow mesenchymal stem cells (BM-MSCs) have been identified as a key candidate for cell therapy. However, their clinical application is restricted by the invasive procedures by which they are obtained, their decrease in proliferation and differentiation capacity with increasing age, and the difficulty of using them in the treatment of patients with hereditary diseases (Atala 2006; Stenderup et al. 2003). As a result, other adult and fetal tissues, including adipose tissue, umbilical cord blood, amniotic fluid, and placental tissue, have been studied as alternative sources of stem cells (Kern et al. 2006; Li et al. 2005; Zheng et al. 2008). Although umbilical-cord-blood-derived mesenchymal stem cells of fetal origin have been shown to be multipotent, far fewer MSCs are obtained than when bone marrow is used as the stem cell source. Moreover, it is difficult to isolate them consistently. Additionally, the proliferative capacity of adipose-tissue-derived stem cells is lower than that of BM-MSCs (Haigh et al. 1999; Kang et al. 2004). Thus, a continuing need exists for alternative sources of MSCs for use in medical applications.

The placenta plays an essential role in fetal development, providing nutrition to the fetus and supporting immunological tolerance. Recently, placenta-derived stem cells (PDSCs) of fetal origin have emerged as an alternative source of MSCs for use in regenerative medicine, because of their capacity for self-renewal and multipotent differentiation and their immunomodulatory properties (Felix and Doherty 1979; Li et al. 2005). Furthermore, the placenta contains large numbers of MSCs, and its use for research purposes is not affected by the ethical issues surrounding the use of human embryonic stem cells (Fu et al. 2006; Li et al. 2005, 2007).

The human placenta is composed of the placental disc, chorioamniotic membranes, and umbilical cord. The chorioamniotic membranes contain amniotic epithelial cells (AECs) and mesenchymal cells including amniotic mesenchymal stem cells (AMSCs) and chorionic mesenchymal stem cells (CMSCs) in the collagenous stroma that underlies the amnion epithelium (Kern et al. 2006; Soncini et al. 2007). MSCs derived from the chorionic villi (CV-MSCs) and the Wharton's jelly of the umbilical cord (WJ-MSCs) have also been isolated from placenta (Igura et al. 2004; Portmann-Lanz et al. 2006; Troyer and Weiss 2008). However, as the chorionic villous stroma contains various cell types (stromal fibroblasts, capillary endothelial cells, and macrophages), the isolation of CV-MSCs might be highly complex and ineffective compared with that of other placental MSCs. Furthermore, CV-MSCs and CMSCs are easily contaminated with maternal cells (Zhang et al. 2006).

We have thus considered chorionic-plate-derived mesenchymal stem cells (CP-MSCs; referred to as AMSCs by other researchers) obtained from thicker placental amniotic connective tissue covering the chorionic plate and WJ-MSCs to be more attractive PDSCs in terms of ease of isolation. In view of the complexity of the placenta, CP-MSCs and WJ-MSCs need to be properly characterized. In this study, we have isolated CP-MSCs and WJ-MSCs from the same placentas and characterized their capacity for self-renewal and potential for multipotent differentiation to evaluate their clinical applicability and potential effectiveness as future cell therapies.

Materials and methods

Isolation and cultivation of PDSCs

Human placentas ($n=5$; gestational age: 38–40 weeks) were obtained following uncomplicated elective Caesarean deliveries. The collection and use of the materials were approved by the Institutional Review Board, CHA hospital. All participating women provided written informed consent.

To isolate AECs and CP-MSCs, the placental membranes were separated by blunt dissection from the placental body and washed in Dulbecco's phosphate-buffered saline (DPBS; Gibco, BRL, N.Y., USA) to remove blood. To isolate AECs, the separated placental membranes were cut into small pieces and incubated at 37°C with shaking (175 rpm) with Hanks balanced salt solution (HBSS; Gibco) without calcium or magnesium but containing 0.05% (w/v) trypsin/EDTA solution (Gibco) according to a previously described protocol (Akle et al. 1981). The cells from the first 10 min of digestion were discarded to exclude debris, and the cells from the second 40-min digests were pooled. To isolate CP-MSCs, amniotic connective tissue of placental membranes was carefully harvested by using two slide glasses and then incubated at 37°C with shaking (175 rpm) for 15 min with HBSS containing 1 mg/ml type I collagenase (Sigma-Aldrich, Bornem, Belgium), 1.2 U/ml dispase (Gibco), 2 mg/ml trypsin (Sigma-Aldrich), 65 µg/ml DNase I (Roche, Basel, Switzerland), and 1× penicillin-streptomycin (Gibco). To isolate CV-MSCs, the vessels of the chorionic villi of placenta, except for the chorionic plate and basal plate, were removed, washed thoroughly in DPBS, and cut into small pieces. The tissues were incubated in the enzyme solution used for the isolation of CP-MSCs. In the case of CV-MSCs, the isolated CV-MSCs were treated with RBC lysis buffer (150 mM NH₄Cl, 10 mM KHCO₃, 0.1 mM EDTA, all Sigma) for RBC lysis. To isolate WJ-MSCs from Wharton's jelly, 4-cm segments of umbilical cord were obtained, and the umbilical vein and the two arteries were

removed. After being minced with scissors, the tissues were digested by incubation at 37°C with shaking (175 rpm) for 3 h in HBSS supplemented with 1 mg/ml type I collagenase, 65 µg/ml DNase I (Roche), and 1× penicillin-streptomycin, according to a previously described protocol, with modifications (Karahuseyinoglu et al. 2007). The viability of the isolated PDSCs was determined by trypan blue exclusion. The PDSCs were cultured in α -modified minimal essential medium (α -MEM; Gibco) supplemented with 10% fetal bovine serum (FBS; Gibco), 25 ng/ml fibroblast growth factor-4 (FGF-4; Sigma-Aldrich), 1 µg/ml heparin (Sigma-Aldrich), and 1× penicillin-streptomycin at 37°C in a humidified atmosphere containing 5% CO₂.

Immunofluorescent staining

The various isolated PDSCs were grown in 8-well chamber slides coated with 5 µg/ml type IV collagen (Sigma-Aldrich). The cells were fixed with 4% paraformaldehyde (PFA; USB, Cleveland, Ohio, USA) for 30 min and permeabilized with 0.25% Triton X-100 (Sigma-Aldrich) for 2 min. The cells were blocked in Dako protein block serum-free (Dako, High Wycombe, UK) for 30 min at room temperature. The cells were incubated with primary antibodies against monoclonal anti-cytokeratin 19 (Sigma-Aldrich), polyclonal anti-vimentin (Sigma-Aldrich), monoclonal anti-cytokeratin 7 (Dako), or monoclonal anti-CD31 (Dako) diluted in an antibody diluent that reduced background effects (Dako) for 1 h. The cells were incubated with normal mouse IgG as a negative control. The cells were then incubated in Alexa Fluor 568 chicken anti-mouse IgG (Invitrogen) or Alexa Fluor 568 donkey anti-goat IgG (Invitrogen) as a secondary antibody for 30 min. The nuclei were counterstained with Prolong gold antifade reagent with 4,6-diamidino-2-phenylindole (DAPI; Invitrogen).

Doubling-time analysis

To examine their doubling-time, the cells were seeded at a density of 5×10^3 cells/cm², and the doubling-time (TD) of the harvested cells was calculated by using an algorithm available online (<http://www.doubling-time.com>):

$$TD = t \times \lg 2 / (\lg N_t - \lg N_0)$$

where N_0 is the number of cells inoculated, N_t is the number of cells harvested, and t is the culture time in hours.

Reverse transcription with the polymerase chain reaction

Total RNA was extracted from cells growing in 6-well plates with the TRIzol LS Reagent (Invitrogen, Carlsbad, Calif., USA). cDNA was synthesized by reverse transcription (RT) from total RNA (1 µg) by using SuperScript III

reverse transcriptase (Invitrogen) according to the manufacturer's instructions. Polymerase chain reaction (PCR) amplification was performed with specific primers (Table 1) in 25-µl reactions containing 100 ng cDNA template, 2.5 U/µl DNA polymerase (Solgent, South Korea), and 200 µM dNTP. β -Actin was used as an internal control. cDNA was subjected to 35 cycles of amplification (20 s at 95°C, 40 s at the appropriate annealing temperature as given in Table 1, and 1 min at 72°C). Amplified PCR products were electrophoresed on 1.5% agarose gels containing 1.5 µg/ml ethidium bromide and visualized under UV light.

Real-time PCR

Real-time PCR analysis was used to quantify differences in gene expression. Target gene expression was normalized to that of an internal reference (18S rRNA). Real-time PCR was performed with primers (Table 1) designed by Bioneer (Daejeon, South Korea) or as referenced in previous studies (Hou et al. 2009; Lee et al. 2010a; Zheng et al. 2008) and SYBR Premix Ex *Taq* (Takara, Japan) in an Exicycler 96 real-time PCR machine (Bioneer). Target sequences were amplified by using the following thermal conditions: 2 min at 95°C, and 40 cycles of 5 s at 95°C and 30 s at 60°C. All reactions were performed in triplicate.

Flow cytometry

To phenotype cell-surface antigens, third-passage cells were stained with monoclonal antibodies specific for the following proteins: CD34-PE, CD45-FITC, CD44-PE, CD90-PE, HLA-ABC-FITC, and HLA-DR-FITC (BD Bioscience, San Jose, Calif., USA), CD31-APC (eBioscience, San Diego, Calif., USA), CD13-PE (BioLegend, San Diego, Calif., USA), CD105-FITC (R&D Systems, Abingdon, UK), and HLA-G (Abcam, Cambridge, UK). After being stained, the cells were washed in DPBS and fixed in 1% PFA. Cells were also treated with appropriate isotype control antibodies (BD Biosciences). Stained cells were analyzed by using a FACSCalibur flow cytometer (Becton Dickinson, N.J., USA). For each sample, at least 10,000 events were acquired.

Differentiation procedures

Mesodermal lineage: adipogenic and osteogenic differentiation

To induce adipogenic differentiation, third-passage CP-MSCs and WJ-MSCs were plated at a density of 5×10^3 cells/cm² in adipogenic induction medium containing 1 µM dexamethasone, 0.5 mM isobutyl methylxanthine (IBMX), 0.2 mM indomethacin, 1.7 µM insulin (all from Sigma-

Table 1 Primer sequences (RT reverse transcription, PCR polymerase chain reaction, F forward, R reverse, TM annealing temperature)

Markers	Gene	Sequence (5' to 3')	TM (°C)	Size (bp)
RT-PCR				
MSC	Oct-4	F: AGT GAG AGG CAA CCT GGA GA R: GTG AAG TGA GGG CTC CCA TA	54	273
	Nanog	F: TTC TTG ACT GGG ACC TTG TC R: GCT TGC CTT GCT TTG AAG CA	54	260
	Sox2	F: GGG CAG CGT GTA CTT ATC CT R: AGA ACC CCA AGA TGC ACA AC	52	200
	NF-68	F: GAG TGA AAT GGC ACG ATA CCT A R: TTT CCT CTC CTT CTT CTT CAC CTT C	58	500
	Cardiac actin	F: GGA GTT ATG GTG GGT ATG GGT C R: AGT GGT GAC AAA GGA GTA GCC A	58	500
	AFP	F: AGC TTG GTG GAT GAA AC R: TCC AAC AGG CCT GAG AAA T	50	200
	HLA-G	F: GCG GCT ACT ACA ACC AGA GC R: GCA CAT GGC ACG TGT ATC TC	58	550
	TERT	F: GAG CTG ACG TGG AAG ATG AG R: CTT CAA GTG CTG TCT GAT TCC AAT G	55	300
	Internal control	β -Actin	F: TCC TTC TGC ATC CTG TCA GCA R: CAG GAG ATG GCC ACT GCC GCA	58
Real-time PCR				
Adipogenic	PPAR γ	F: TTG ACC CAG AAA GCG ATT CC R: AAA GTT GGT GGG CCA GAA TG	60	101
	Adipsin	F: GGT CAC CCA AGC AAC AAA GT R: CCT CCT GCG TTC AAG TCA TC	60	272
Osteogenic	Col I	F: AGA CAT CCC ACC AAT CAC CT R: CGT CAT CGC ACA ACA CCT	60	119
Hepatogenic	Albumin	F: TGA GTT TGC AGA AGT TTC CA R: CCT TTG CCT CAG CAT AGT TT	60	293
	HNF1 α	F: TAC ACC ACT CTG GCA GCC ACA CT R: CGG TGG GTA CAT TGG TGA CAG AAC	60	114
	α 1-AT	F: TCG CTA CAG CCT TTG CAA TG R: TTG AGG GTA CGG AGG AGT TCC	60	142
	CYP 3A4	F: ATC ATT GCT GTC TCC AAC CTT CAC R: TGC TTC CCG CCT CAG ATT TCT C	60	103
Internal control	18S rRNA	F: GTA ACC CGT TGA ACC CCA TT R: CCA TCC AAT CGG TAG TAG CG	60	151

Aldrich), 10% FBS, and 1 \times penicillin-streptomycin. The growth medium was replaced twice a week. After 21 days, the cells were fixed with 4% PFA and incubated for 1 h with Oil Red O (Sigma-Aldrich), which stains lipids, to visualize lipid vesicles. Nuclei were counterstained with Mayer's hematoxylin (Sigma-Aldrich) for 1 min.

To induce osteogenic differentiation, third-passage CP-MSCs and WJ-MSCs were plated at a density of 5 \times 10³ cells/cm² in osteogenic induction medium containing 1 μ M dexamethasone, 10 mM glycerol 2-phosphate, 50 μ M L-ascorbic acid 2-phosphate (all from Sigma-Aldrich), 10% FBS, and 1 \times penicillin-streptomycin. The growth medium was replaced twice a week. After 21 days, the cells were subjected to von Kossa staining, namely incubation for 1 h

with 5% silver nitrate (Sigma-Aldrich) under light, to evaluate their accumulation of calcium deposits.

Endodermal lineage: hepatogenic differentiation

Fourth-passage cells were seeded at a density of 5 \times 10³ cells/cm² in low-glucose Dulbecco's modified Eagle medium (DMEM; Gibco) supplemented with 40% MCDB 201 medium (Sigma-Aldrich), 2% FBS, and 1 \times penicillin-streptomycin in 6-well or 24-well plates containing coverslips coated with 5 μ g/ml type IV collagen. After 48 h, the growth medium was replaced with low-glucose DMEM supplemented with 20 ng/ml epidermal growth factor (EGF; Peprotech EC), 10 ng/ml basic fibroblast growth factor

(bFGF; Sigma-Aldrich), 10 ng/ml bone morphogenetic protein-4 (BMP-4; Peptide EC), 40% MCDB 201 medium, and 1× penicillin-streptomycin, and the cells were cultured for a further 48 h. Next, differentiation was induced by incubating the cells for 7 days with step-1 medium consisting of low-glucose DMEM supplemented with 20 ng/ml hepatocyte growth factor (HGF; Peptide EC), 10 ng/ml bFGF, 40% MCDB 201 medium, 2% FBS, and 1× penicillin-streptomycin. Cell maturation was then induced through incubation for 7 days in step-2 medium consisting of low-glucose DMEM supplemented with 20 ng/ml oncostatin M (Peptide EC), 1 μM dexamethasone, 1× ITS+ Premix (universal culture supplement, which contains insulin, human transferrin, and selenous acid), 40% MCDB 201 medium, 2% FBS, and 1× penicillin-streptomycin. During steps 1 and 2, the culture medium was replaced every 3 days. After 18 days, the cells were incubated for 1 h at 37°C with 1 mg/ml indocyanine green (ICG; Dong in Dang Pharm., Korea), the uptake of which was then evaluated.

Statistics

Data are presented as means±standard error of the mean (SEM). The results were analyzed by using PASW statistics software (SPSS, Chicago, Ill., USA). Within-group differences in gene expression were identified by using the Mann-Whitney U-test, and between-group differences were detected by using the Wilcoxon rank sum test. Values of $P < 0.05$ were considered to be statistically significant.

Results

Cell types amongst various PDSCs

PDSCs including AECs, CP-MSCs, CV-MSCs, and WJ-MSCs were isolated from normal term placentas. The morphology of the isolated AECs showed typical squamous cells (Fig. 1a). Otherwise, the morphologies of the primary CP-MSCs, CV-MSCs, and WJ-MSCs were spindle-like and fibroblastoid (Fig. 1a'–a'''). To demonstrate the cell types amongst the various PDSCs, immunofluorescent staining was performed with several antibodies well known as cell-type-specific markers. AECs were strongly positive for anti-cytokeratin 19 and anti-cytokeratin 7 for epithelial cell markers (Fig. 1b, d). However, CP-MSCs, CV-MSCs, and WJ-MSCs were strongly positive for anti-vimentin as a mesenchymal cell marker (Fig. 1c'–c'''), although weak reaction in AECs was also observed by immunofluorescent staining (Fig. 1c). In CV-MSCs, positive cells for anti-cytokeratin 7 (Fig. 1d''), a marker of trophoblasts and for anti-CD31 (Fig. 1e''), a marker of endothelial cells, were

observed. These results show that CP-MSCs and WJ-MSCs have more pure cell types as an MSC source, whereas AECs are mostly epithelial cells, and several cell types in addition to MSCs are mixed together in CV-MSCs.

Characterization of CP-MSCs and WJ-MSCs

We characterized CP-MSCs and WJ-MSCs among the various PDSCs. Live cell yields were $4.5 \pm 2.7 \times 10^4$ for CP-MSCs and $6.4 \pm 3.2 \times 10^4$ for WJ-MSCs per gram of wet tissue (placental amnion and umbilical cord, respectively; $n=5$). The morphology of the primary CP-MSCs and WJ-MSCs was spindle-like and fibroblastoid (Fig. 2a, b).

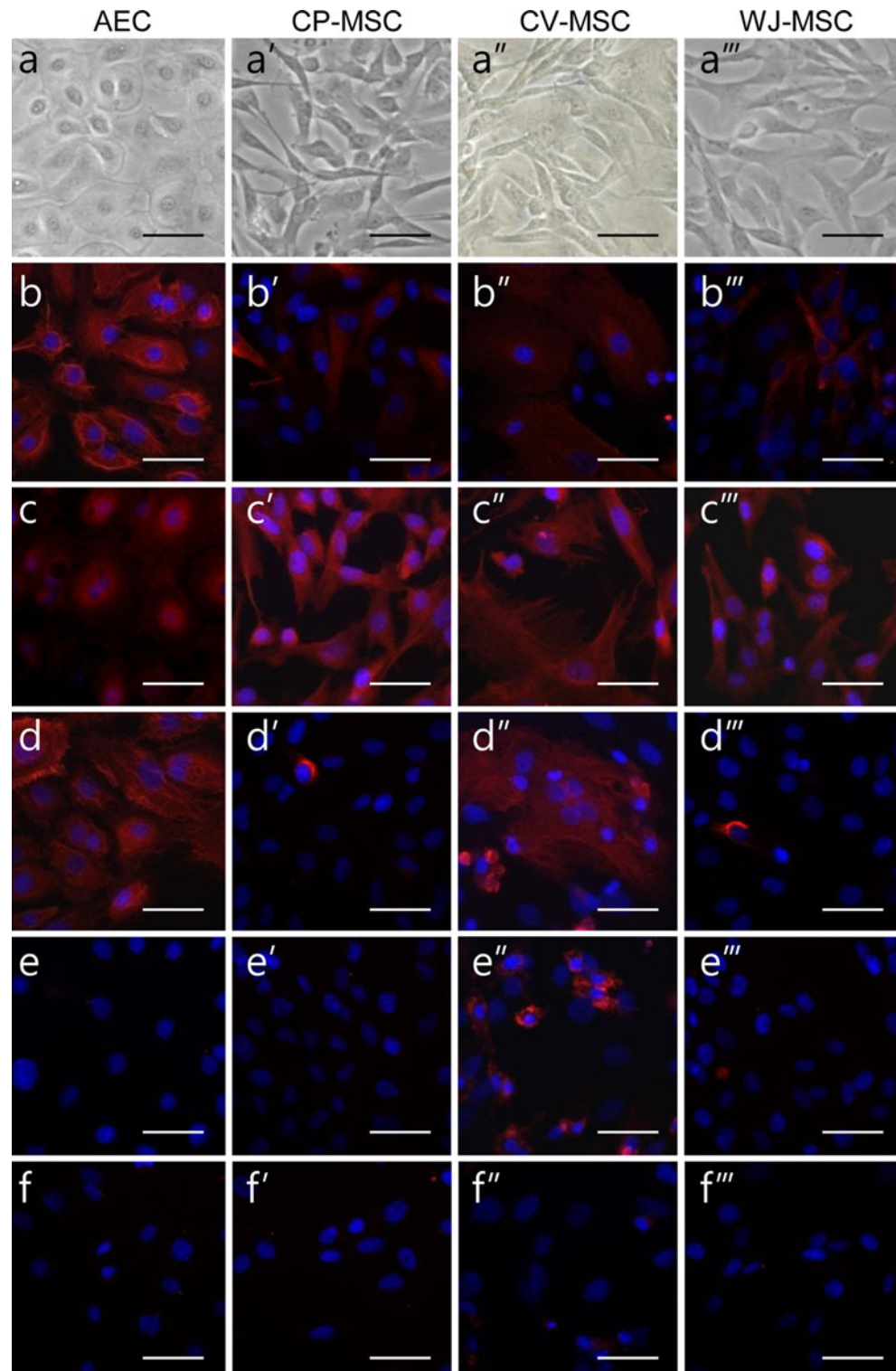
To analyze proliferation rates, we calculated doubling-times for third- to eleventh-passage CP-MSCs and WJ-MSCs (Fig. 2c). The doubling-times for CP-MSCs and WJ-MSCs, respectively, were 27 ± 8 h and 21 ± 0 h at passage three and 42 ± 7 h and 32 ± 6 h at passage eleven. The proliferative potential of WJ-MSCs tended to be higher than that of CP-MSCs at all passages. The results of RT-PCR analyses showed that both CP-MSCs and WJ-MSCs expressed self-renewal and pluripotent stem cell markers such as (Oct4), Nanog, SRY-related HMG-box 2 (Sox2), and telomerase reverse transcriptase (TERT), plus the germinal layer markers NF68, Cardiac, and AFP (Fig. 2d). Furthermore, mRNA for HLA-G, which has immunomodulatory effects, was expressed in both cell types (Fig. 2d).

The immunophenotypes of CP-MSCs and WJ-MSCs were analyzed by flow cytometry (Fig. 2e). CP-MSCs and WJ-MSCs were positive for the mesenchymal stem cell markers CD90, CD44, CD13, and CD105, and negative for hematopoietic markers and endothelial marker such as CD45, CD34, and CD31. Additionally, the presence of human leukocyte antigen receptor (HLA)-ABC (MHC class I) in CP-MSCs and WJ-MSCs and the absence of HLA-DR (MHC class II) were consistent with the general description of MSCs. Of CP-MSCs and WJ-MSCs, $12 \pm 11\%$ and $9 \pm 6\%$ were positive for HLA-G, respectively. These findings show that CP-MSCs and WJ-MSCs possess both the capacity for self-renewal and immunomodulatory properties.

Differentiation of CP-MSCs and WJ-MSCs to mesodermal-lineage cells

To investigate their capacity for mesodermal differentiation, CP-MSCs and WJ-MSCs were induced to undergo adipogenic and osteogenic differentiation (Figs. 3, 4). Cells derived from both CP-MSCs and WJ-MSCs as a result of adipogenic differentiation bore the appearance of adipocytes (Fig. 3a, b). The accumulation of lipid vacuoles in CP-MSCs and WJ-MSCs was demonstrated by Oil Red O staining (Fig. 3c, d). The mRNA expression of adipogenesis-specific markers in CP-MSCs and WJ-MSCs

Fig. 1 Marker expression according to cell types amongst various placenta-derived stem cells (PDSCs) isolated from human placenta. PDSCs, i.e., amniotic epithelial cells (AECs), chorionic-plate-derived mesenchymal stem cells (CP-MSCs), chorionic villi MSCs (CV-MSCs), and Wharton's jelly-derived MSC (WJ-MSCs) were isolated from various components of placenta. **a-a'''** Morphology of various isolated PDSCs. The isolated PDSCs were determined with anti-cytokeratin 19 as an epithelial cell marker (**b-b'''**), anti-vimentin as a mesenchymal cell marker (**c-c'''**), cytokeratin 7 as epithelial cell and trophoblast markers (**d-d'''**), and anti-CD31 as an endothelial cell marker (**e-e'''**) by immunofluorescent staining. Normal mouse IgG (**f-f'''**) was used as a negative control. Nuclei were counterstained with DAPI (blue). Bars 50 μ m



was analyzed by real-time PCR (Fig. 3e, f). Levels of adipsin (Fig. 3e) and peroxisome-proliferator-activated receptor γ (PPAR γ) (Fig. 3f) mRNA expression were higher in CP-MSCs and WJ-MSCs induced to undergo adipogenic differentiation than in undifferentiated cells ($P<0.05$). The expression of adipsin and PPAR γ mRNA

was significantly higher in CP-MSCs induced to undergo differentiation than in similarly treated WJ-MSCs (Fig. 3e, f; $P<0.05$). CP-MSCs displayed a higher expression of adipogenesis-related genes than WJ-MSCs, although no difference was seen in the accumulation of lipid vacuoles between the two cell types. These results indicate that CP-

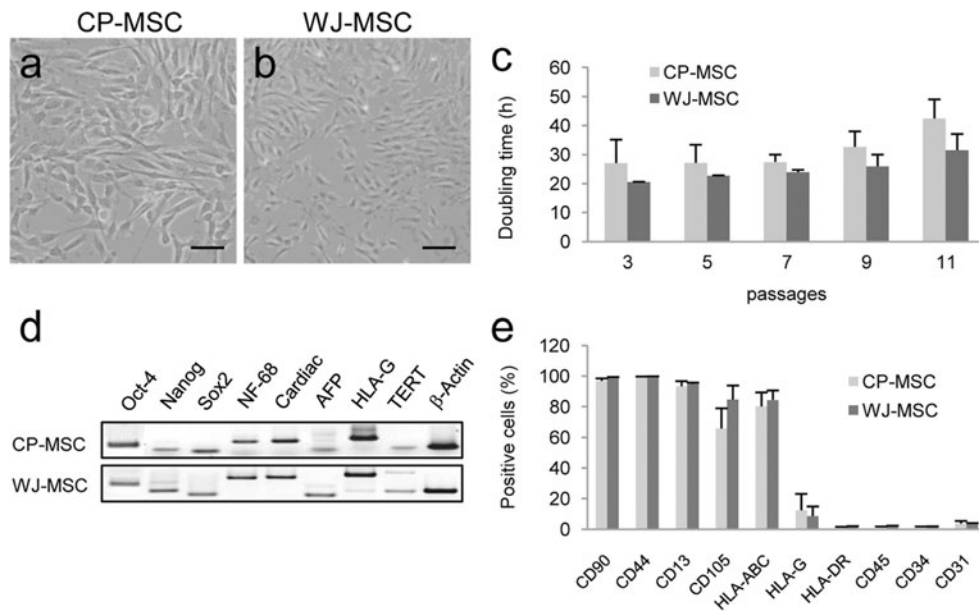


Fig. 2 Characterization of CP-MSCs and WJ-MSCs. **a, b** Morphology of primary CP-MSCs and WJ-MSCs. Bars 50 μ m. **c** Doubling-time analysis of third- to eleventh-passage CP-MSCs and WJ-MSCs. **d** Analysis by reverse transcription with the polymerase chain reaction (RT-PCR) of the self-renewal markers Oct-4, Nanog, Sox-2, and TERT, the germinal layer markers NF-68, Cardiac, and AFP, and the immunomodulatory marker HLA-G. β -Actin was used as an internal

control. **e** Immunophenotypes of CP-MSCs and WJ-MSCs by using flow cytometry. Third-passage CP-MSCs and WJ-MSCs were positive for CD90, CD44, CD13, and CD105, and negative for CD45, CD34, and CD31. Data for HLA-ABC, HLA-DR, and HLA-G are also shown. Percentages of cells positive for these different cell surface markers were calculated by comparison of target protein signals with those for isotype controls

MSCs have a stronger potential for adipogenic differentiation than WJ-MSCs.

CP-MSCs and WJ-MSCs induced to undergo osteogenic differentiation became progressively broader following the induction of differentiation (Fig. 4a, b). The result of von

Kossa staining showed that CP-MSCs and WJ-MSCs differentiated into osteogenic cells, and that WJ-MSCs accumulated more mineralized matrix than CP-MSCs (Fig. 4c, d). To confirm osteogenic differentiation, the expression of the osteogenesis-related gene for type I collagen (Col I) in CP-

Fig. 3 Adipogenic differentiation of CP-MSCs and WJ-MSCs. **a, b** Morphology of CP-MSCs and WJ-MSCs grown in adipogenic medium. **c, d** Accumulation of oil droplets in differentiated CP-MSCs and WJ-MSCs, determined by Oil-Red O staining. Cells were counterstained with hematoxylin. Bars 50 μ m. Adipogenic differentiation of CP-MSCs and WJ-MSCs was confirmed by the detection of mRNA encoding the adipogenic proteins adipisin (e) and PPAR γ (f) by real-time PCR (- undifferentiated cells, + differentiated cells). * $P < 0.05$, within-group differences; ** $P < 0.05$, between-group differences

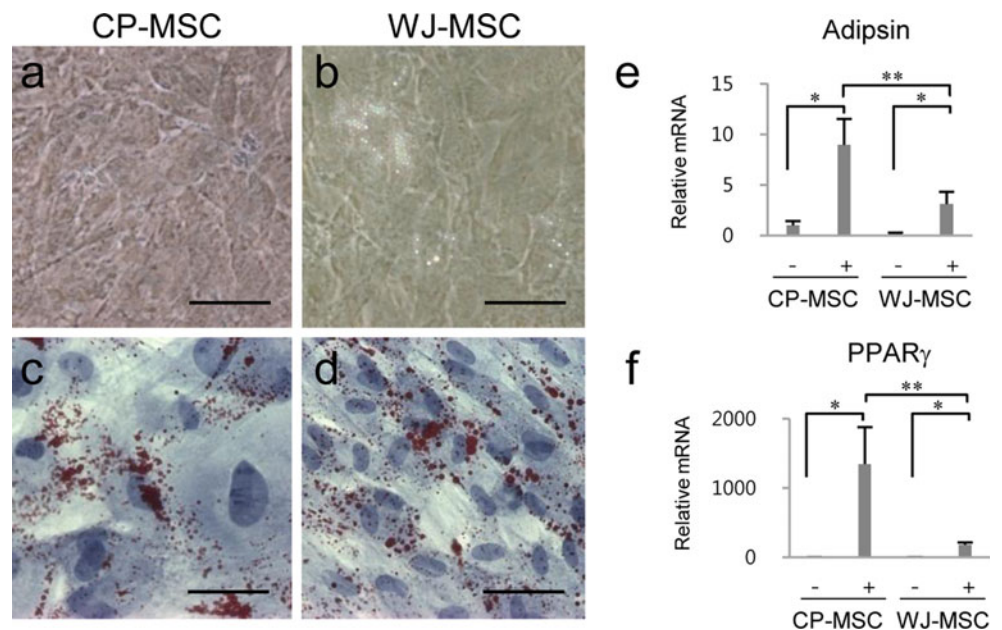
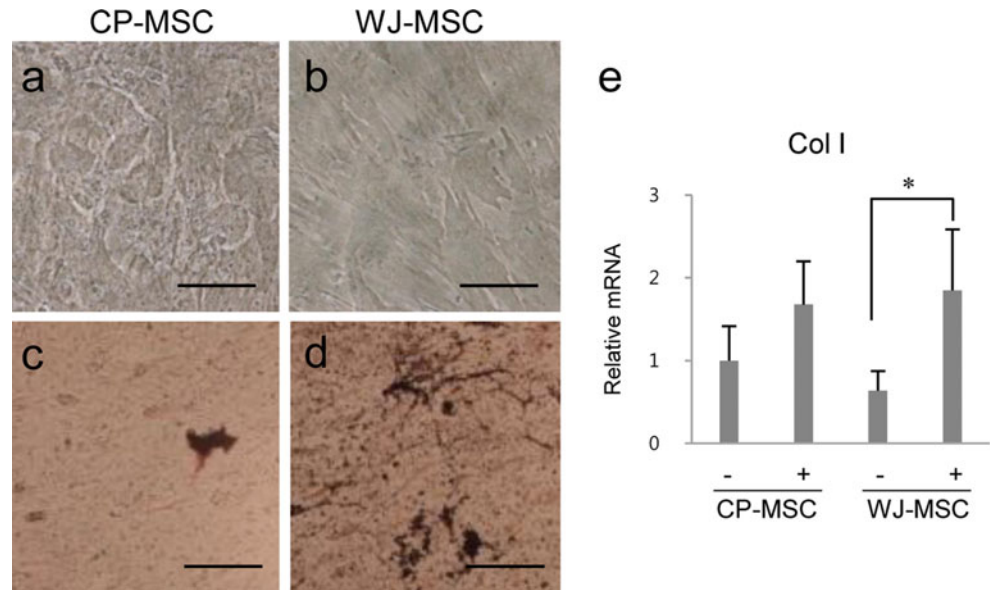


Fig. 4 Osteogenic differentiation of CP-MSCs and WJ-MSCs. **a, b** Morphologies of CP-MSCs and WJ-MSCs grown in osteogenic medium. **c, d** Calcium deposition in CP-MSCs and WJ-MSCs was evaluated by von Kossa staining. **Bars** 50 μ m. **e** Osteogenic differentiation of CP-MSCs and WJ-MSCs was confirmed by the detection of mRNA encoding the osteogenic protein type I collagen (*Col I*) by real-time PCR (- undifferentiated cells, + differentiated cells). * $P < 0.05$, within-group differences



MSCs and WJ-MSCs was assessed by real-time PCR (Fig. 4e). Col I mRNA expression was higher in CP-MSCs and WJ-MSCs induced to undergo osteogenic differentiation than in undifferentiated cells. Notably, Col I mRNA expression was significantly higher in WJ-MSCs induced to undergo osteogenic differentiation than in undifferentiated WJ-MSCs ($P < 0.05$); however, the differences between the two cell types were not statistically significant. These results indicate that

WJ-MSCs possibly have a stronger capacity for osteogenic differentiation than CP-MSCs.

Differentiation of CP-MSCs and WJ-MSCs to endodermal-lineage cells

To induce their hepatogenic differentiation, CP-MSCs and WJ-MSCs were cultured in hepatogenic differentiation

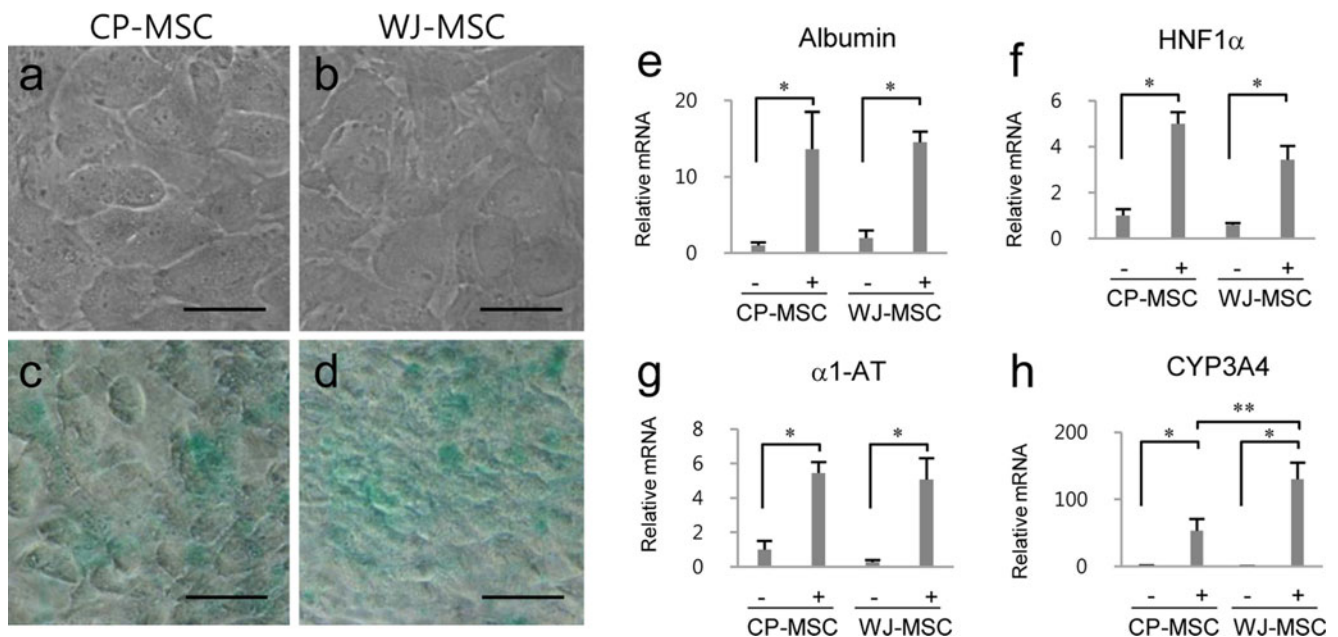


Fig. 5 Hepatogenic differentiation of CP-MSCs and WJ-MSCs. **a, b** Morphology of CP-MSCs and WJ-MSCs grown in hepatogenic medium. **c, d** Hepatic differentiation of CP-MSCs and WJ-MSCs was evidenced by the uptake of indocyanine green (ICG). **Bars** 50 μ m. **e-h** Hepatogenic differentiation of CP-MSCs and WJ-MSCs was confirmed by the

detection of mRNA encoding the hepatogenic proteins and transcription factors albumin, hepatocyte nuclear factor 1 α (*HNF1 α*), α 1-antitrypsin (*α 1-AT*), and cytochrome P450 3A4 (*CYP3A4*) by real-time PCR (- undifferentiated cells, + differentiated cells). * $P < 0.05$, within-group differences; ** $P < 0.05$, between-group differences

media. Culture for 7 days in step-1 hepatogenic medium produced no obvious changes in cell morphology. However, subsequent culture for 7 days in step-2 medium containing oncostatin M yielded cells with a cuboidal morphology, characteristic of hepatocyte-like cells (Fig. 5a, b). To determine whether these cells were indeed functional hepatocytes, their ability to take up ICG (Fig. 5c, d) was evaluated. The capacity of WJ-MSCs to take up ICG was stronger than CP-MSCs. To confirm hepatogenic differentiation, the mRNA expression of specific hepatogenic markers was examined in CP-MSCs and WJ-MSCs by real-time PCR (Fig. 5e–h). The mRNA expression of the specific hepatogenic markers albumin (Fig. 5e), hepatocyte nuclear factor 1 α (HNF1 α ; Fig. 5f), α 1-antitrypsin (α 1-AT; Fig. 5g), and cytochrome P450 3A4 (CYP3A4; Fig. 5h) was significantly higher in CP-MSCs and WJ-MSCs induced to undergo hepatogenic differentiation than in undifferentiated cells ($P < 0.05$). Notably, CYP3A4 mRNA levels for mature hepatocyte marker were significantly higher in WJ-MSCs induced to undergo hepatogenic differentiation than in similarly treated CP-MSCs (Fig. 5h; $P < 0.05$) although real-time PCR revealed no significant difference in albumin, HNF1 α , and α 1-AT mRNA expression levels in CP-MSCs and WJ-MSCs induced to undergo hepatogenic differentiation (Fig. 5e–g). These results indicate that WJ-MSCs display a stronger expression of mRNA encoding CYP3A4, a marker of mature hepatocytes, and that they might have a stronger capacity for hepatogenic differentiation than CP-MSCs.

Discussion

PDSCs are emerging as promising candidates for cell therapy in regenerative medicine because of their multipotency, ease of isolation, and abundance (Parolini et al. 2008; Troyer and Weiss 2008). Various PDSCs including AECs, CP-MSCs, CV-MSCs, and WJ-MSCs have been isolated from normal-term placentas and characterized. In this study, we have observed that the morphology of subcultured AECs changes from epithelial-like to mesenchymal-like (data not shown), suggestive of an epithelial-mesenchymal transition as in a previous report (Bilic et al. 2008). We have confirmed that several cell types such as trophoblasts, endothelial cells, and MSCs are mixed in CV-MSCs (Haigh et al. 1999; Miettinen et al. 1994). Additionally, CP-MSCs and WJ-MSCs have normal karyotypes and do not cause teratoma formation (data not shown; Miki and Strom 2006). Furthermore, a karyotype analysis of CP-MSCs and WJ-MSCs from a male neonate has indicated that the isolated cells originate from the fetus (data not shown); on the other hand, several researchers have reported that CV-MSCs are contaminated with

maternal cells (Soncini et al. 2007; Zhang et al. 2006). Therefore, we consider that the characterization of CP-MSCs and WJ-MSCs with a more pure cell type for MSCs originating from the fetus and an assessment of their differentiation potential are important for future studies into the applicability and effectiveness of PDSCs in cell therapy.

CP-MSCs and WJ-MSCs have a similar morphology, gene expression pattern, capacity for self-renewal, and cell surface immunophenotype. As shown in Fig. 2a, b, the morphology of primary CP-MSCs and WJ-MSCs is similar to that of other MSCs, being fibroblastoid and spindle-like (Short et al. 2003). Naïve CP-MSCs and WJ-MSCs also express the germinal layer makers NF68, Cardiac, and AFP, and the pluripotent stem cell markers, Oct4, Nanog and Sox2, as reported by others (Alviano et al. 2007; Weiss et al. 2006). Additionally, TERT mRNA is expressed in both cell types. However, telomerase activity has not been detected in either cell type in an assay with the telomere repeat amplification protocol (data not shown). Consistent with our results, the expression of the RNA component of human telomerase has previously been detected in the chorion and does not correlate with telomerase activity (Kyo et al. 1997). The expression of pluripotent stem cell markers in CP-MSCs and WJ-MSCs might enhance their desirability as a stem cell source. According to our data, the immunophenotypes of CP-MSCs and WJ-MSCs are similar to those of other MSCs. hAMSCs (Steigman et al. 2008) and hWJMSCs (Bakhshi et al. 2008) have been shown to be positive for CD29, CD73, and CD166. Additionally, we have demonstrated the similar biological characteristics of CP-MSCs and WJ-MSCs until passage 9 with respect to their morphology and immunophenotype (data not shown). Weiss et al. (2006) have reported that CD105 and CD49 are highly expressed in 74%–97% of fourth-passage WJ-MSCs but in only 15%–25% of eighth-passage WJ-MSCs. Consistent with previous reports, we have found CD105 to be highly expressed in 66% of third-passage CP-MSCs and 85% of third-passage WJ-MSCs; however, we have also observed that approximately 60% of both cell types at passage 9 display CD105 expression (data not shown). These results show that CP-MSCs and WJ-MSCs have self-renewal potentials.

CP-MSCs and WJ-MSCs express the non-polymorphic and non-classical HLA-G at the mRNA and protein levels. Banas et al. (2008) have reported that the expression of HLA-G on cultured amniotic mesenchymal progenitor cells increases following their exposure to interferon- γ . HLA-G produces inhibitory effects by directly binding to inhibitory receptors on natural killer (NK) cells and other leukocytes. It also induces apoptosis in activated CD8⁺ T cells and inhibits CD4⁺ T cell proliferation (Hunt et al. 2005). Furthermore, HLA-G has been shown to interact only with

certain NK cell inhibitory receptors, including killer inhibitory receptor, which allows inhibition at the maternal-fetal interface to be controlled (Gonen-Gross et al. 2010). Therefore, we suggest that CP-MSCs and WJ-MSCs with an immunosuppressive effect possess potentially important characteristics of transplantable stem cells.

To isolate CP-MSCs, we first harvested connective tissue and digested it with an enzyme mixture. This approach yielded an almost pure CP-MSC population. In contrast, CP-MSCs obtained by digestion with collagenase after the removal of amniotic epithelial cells (AECs) with trypsin were previously shown to be inevitably contaminated with AECs. To isolate WJ-MSCs, we shortened the enzyme digestion time to 3 h by applying agitation. Other researchers digested Wharton's jelly tissue by incubation with 1 mg/ml type I collagenase for 18–24 h at 37°C or by using other enzyme mixtures containing trypsin (Fu et al. 2006; Hou et al. 2009). However, our aim was to isolate uncontaminated cells that were not damaged as a result of prolonged exposure to enzymes.

The biological properties of CP-MSCs and WJ-MSCs are generally similar. Nevertheless, WJ-MSCs, which can be isolated from Wharton's jelly of the umbilical cord, can be obtained in larger numbers than CP-MSCs, which are isolated from the connective tissue of placental membranes. hAMSCs and WJ-MSCs, as cells of fetal origin, are reported to have higher proliferative capacities than BM-MSCs. However, whether hAMSCs or WJ-MSCs have the higher proliferation capacity is difficult to judge (Alviano et al. 2007; Felix and Doherty 1979). In our study, the proliferation capacity of WJ-MSCs tended to be higher than that of CP-MSCs. In addition, the doubling-time of WJ-MSCs at passage 16 was approximately 42 h (data not shown), that of CP-MSCs at the same passage was over 100 h. Thus, WJ-MSCs have the potential to generate larger numbers of cells than CP-MSCs and may be a more promising candidate for clinical applications.

We further compared the capacities of CP-MSCs and WJ-MSCs for multipotent differentiation. We first tested their ability to differentiate into mesodermal-lineage cells (adipogenic and osteogenic cells). We showed that CP-MSCs displayed higher expression of genes encoding the adipogenic proteins adiponectin and PPAR γ than WJ-MSCs and appeared to possess a stronger capacity for adipogenic differentiation, although no difference was seen in the accumulation of lipid vacuoles in the two cell types. CP-MSCs and WJ-MSCs also displayed the ability to undergo osteogenic differentiation, consistent with previous reports (Hou et al. 2009; Kern et al. 2006; Yen et al. 2005). Our data indicated that WJ-MSCs accumulated more mineralized matrix than CP-MSCs and displayed a greater potential for osteogenic differentiation, although no significant difference was observed in the expression of genes

encoding the osteogenic proteins type I collagen between the two cell types. Recently, Hou et al. (2009) have shown that WJ-MSCs have a phenotype and a capacity for BMP2-induced osteogenic differentiation similar to those of BM-MSCs in vitro. We have confirmed that CP-MSCs and WJ-MSCs have a capacity for chondrogenic differentiation, expressing aggrecan and cartilage oligomeric matrix protein (COMP) mRNA and yielding a matrix-rich and multilayered mass accompanied by the accumulation of sulfated proteoglycans deposited by chondrocytes, as evidenced by Alcian blue staining (data not shown). Various researchers have reported that CP-MSCs and WJ-MSCs induced to undergo hepatogenic differentiation express hepatic markers, store glycogen, and produce urea (Fu et al. 2006; Tamagawa et al. 2007), although the cell type that had the better potential for hepatogenic differentiation was not determined. Consistent with these studies, our data show that CP-MSCs and WJ-MSCs are able to differentiate into hepatocyte-like cells. Interestingly, WJ-MSCs have been found to have a stronger capacity to take up ICG than CP-MSCs, and the expression of mRNA encoding CYP3A4, a marker of mature hepatocytes, is significantly higher in WJ-MSCs induced to undergo hepatogenic than in similarly treated CP-MSCs. Our data suggest that WJ-MSCs have a stronger capacity for hepatogenic differentiation than CP-MSCs. We have previously reported that CP-MSCs engrafted onto CCl₄-injured rat livers differentiate into hepatocyte-like cells in vivo (Lee et al. 2010b). Moreover, the direct injection of human umbilical mesenchymal stem cells (HUMSCs), isolated from Wharton's jelly, into CCl₄-injured rat livers has been shown to reduce liver fibrosis significantly. However, they did not express albumin or alpha-fetoprotein, two markers of differentiated hepatocytes (Tsai et al. 2009). Although both cell types have been found to be effective at treating liver fibrosis in a rat model, a comparison of their efficacies is difficult, because of the differences in the transplantation conditions. Thus, further in vivo studies are required to determine the capacity of both cell types to undergo hepatogenic differentiation. We have also confirmed that CP-MSCs and WJ-MSCs at passage 9 have potentials for adipogenic and hepatogenic differentiation and for the accumulation of lipid vacuoles and uptake of ICG, respectively (data not shown). On the other hand, we have found it difficult to observe accumulated mineralized matrix over both cell types at passage 9 when induced into osteogenic differentiation (data not shown). After neural differentiation induction as previously reported (Lee et al. 2004), we have been able to observe that the morphology of both cell types was changed to neural-like cells and that they both express Tuj1 as a neuron marker as demonstrated by immunofluorescent staining (data not shown). In view of the general trend of their capacities, we conclude that CP-MSCs and WJ-MSCs have self-renewal potentials, although some characteristics

change with passage in vitro. Further studies are needed to confirm whether the changes in their characteristics affect the differentiation potential and function of MSCs.

In conclusion, CP-MSCs and WJ-MSCs isolated from normal term placentas have similar biological properties and differentiation potential to produce multi-lineages, however, they differ in terms of their potential to differentiate into different types of cells. Based on our results, we propose that CP-MSCs and WJ-MSCs are useful sources of cells for appropriate clinical applications in the treatment of diseases.

Acknowledgments We thank Dr. Chong Jai Kim (Department of Pathology, Wayne State University School of Medicine, Detroit, Michigan, USA) for critical comments on the manuscript.

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