



Isolation and comparative analysis of potential stem/progenitor cells from different regions of human umbilical cord



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ABSTRACT

Human umbilical cord (hUC) blood and tissue are non-invasive sources of potential stem/progenitor cells with similar cell surface properties as bone marrow stromal cells (BMSCs). While they are limited in cord blood, they may be more abundant in hUC. However, the hUC is an anatomically complex organ and the potential of cells in various sites of the hUC has not been fully explored. We dissected the hUC into its discrete sites and isolated hUC cells from the cord/placenta junction (CPJ), cord tissue (CT), and Wharton's jelly (WJ). Isolated cells displayed fibroblastoid morphology, and expressed CD29, CD44, CD73, CD90, and CD105, and showed evidence of differentiation into multiple lineages in vitro. They also expressed low levels of pluripotency genes, OCT4, NANOG, SOX2 and KLF4. Passaging markedly affected cell proliferation with concomitant decreases in the expression of pluripotency and other markers, and an increase in chondrogenic markers. Microarray analysis further revealed the differences in the gene expression of CPJ-, CT- and WJ-hUC cells. Five coding and five lncRNA genes were differentially expressed in low vs. high passage hUC cells. Only MAEL was expressed at high levels in both low and high passage CPJ-hUC cells. They displayed a greater proliferation limit and a higher degree of multi-lineage differentiation in vitro and warrant further investigation to determine their full differentiation capacity, and therapeutic and regenerative medicine potential.

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1. Introduction

The successful isolation and culture of human embryonic stem cells (ESCs) was a landmark achievement, which generated significant interest in the field of stem cell biology. Pluripotent ESCs have the ability to self-renew indefinitely and to differentiate into ectoderm, mesoderm and endoderm (Carpenter et al., 2003; Hoffman and Carpenter, 2005; Itskovitz-Eldor, 2002). While they are the most primitive, both ethical and technical issues limit their therapeutic use (Fischbach and Fischbach, 2004). Furthermore, their potential to form teratomas is also a major concern (Goldring et al., 2011). These challenges prompted studies to search for alternative sources of stem cells that do not pose moral, ethical and safety dilemmas. Studies have shown the presence of adult stem cells (ASCs) in various organs and body tissues. Bone marrow (BM) has been a well-known source of ASCs, particularly hematopoietic stem cells (HSCs), and to a lesser extent skeletal stem cells [SSCs, also

known as bone marrow-derived mesenchymal stem cells (Bianco and Robey, 2015)] and endothelial progenitors. BM-derived HSCs have been widely used for transplantation therapy to treat leukemia and blood disorders. These cells have the ability to self-renew, are multipotent and are able to differentiate into all blood lineages. Potential applications of bone marrow stromal cells (BMSCs), which contain the subset of SSCs (Robey et al., 2014), are limited because harvesting of BM involves an invasive and painful procedure with possible donor site morbidity. In addition, donor age, genetics, and exposure to environmental stress could cause a significant reduction of biological activity in BMSCs (D'Ippolito et al., 1999). Since a large number of cells are required for cell therapy to regenerate tissues, or to treat most human diseases and dysfunctions, it is imperative to search for new and robust sources of multipotent ASCs.

Recently, human umbilical cord (hUC) has been investigated as a source of cells with stem/progenitor cell properties. hUC is a desirable source of stem/progenitor cells as it is routinely discarded after delivery, collection is non-invasive and an abundant number of cells are present in cord tissue (Catacchio et al., 2013; Dominici et al., 2006; Weiss et al., 2006). The differentiation potential and non-immunogenic nature of hUC-derived cells could make them an ideal source for regenerative

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medicine (Ennis et al., 2008; Weiss et al., 2006). Stem/progenitor cells derived from peri-natal sources such as amniotic fluid, cord blood, cord tissue and placenta are less likely to be altered due to aging or environmental stresses. While the presence of fibroblastic cells with BMSC-like characteristics in cord blood is limited, umbilical cord has been found to be a promising source of potential stem/progenitor cells. However, the cord is an anatomically complex organ and isolation of potential stem/progenitor cells from its various sites has not been rigorously investigated.

In this study, we dissected the hUC into the cord-placenta junction (CPJ), cord tissue (CT), and Wharton's jelly (WJ). Cells isolated from these sites exhibited fibroblastoid morphology, expressed markers found on BMSCs and on other tissue-specific stem/progenitor cells. In vitro, they displayed signs of multipotency. In addition, they expressed pluripotency genes. CPJ-hUC cells showed higher proliferation capabilities as compared with CT- and WJ-hUC cells. These findings suggest that CPJ-hUC cells may have a potential use in tissue engineering and regenerative medicine. Nevertheless, further studies are warranted to fully explore the efficacy and differentiation potential of CPJ-hUC cells.

2. Materials and methods

2.1. Collection and processing of human umbilical cord samples

hUC samples ($n = 50$) were obtained from consented healthy donors through the Beaumont Hospital BioBank, Royal Oak, MI under an HIC (HIC #2012-101) approved protocol. The hUC samples were processed within 2–4 h of delivery. Briefly, the sample was rinsed in PBS several times until all the blood clots were removed. The hUC was then dissected to separate CPJ, (the region between the cord and placenta), CT (the outer layer of the cord), and WJ (the jelly-like tissue within the cord and surrounding the blood vessels). The CPJ, CT and WJ tissues were separately minced into approximately 1–2 mm pieces with the help of surgical and dissection tools, before culturing the explants.

2.2. Isolation of cells

The explants were cultured in 75 cm² culture flasks using culture medium (CM) including DMEM with 4500 mg/ml glucose and 2 mM L-glutamine (Invitrogen), supplemented with 10% FBS (Alekun Biologicals). The medium was changed every 3 days until the cells started to migrate from the explants (5–10 days after plating), after which medium was changed every 1–2 days. When explant cells populated approximately 2/3 of the flask, they were dissociated using TrypLE Select (Invitrogen) and were considered as P0 cells. They were then passaged (P1) in new culture flasks at a concentration of 1×10^4 cells/cm² for amplification. When these cells reached 70% confluency, they were harvested using TrypLE Select, cryopreserved, and sub-cultured for further studies.

2.3. Proliferation assay

Low passage (LP, P2–5) cells derived from CPJ, CT, and WJ were seeded in 24-well plates at a concentration of 5×10^3 cells/well. After predetermined time intervals (1–8 days), triplicate wells were treated with 5 mg/ml MTT solution (Sigma) for 2 h in the dark at 37 °C in 5% CO₂ incubator. The reaction was then stopped and the formazan complex was dissolved using 150 μ l isopropanol with 10 μ l of 0.1 N HCL on a shaker for 15 min in the dark. The optical density in each well was determined at 570 nm using an EPOCH plate reader (BioTek). The background absorbance of blank wells was subtracted from all the measured values.

2.4. Cell cycle analysis

LP cells were subjected to cell cycle analysis. Briefly, cells were grown to 70% confluency, trypsinized, and washed with PBS. Cells (10^6) were fixed with cold absolute ethanol and stored overnight at 4 °C, and then treated with 1 mg/ml RNase and PI staining solution (Sigma) for 40 min in the incubator at 37 °C in the dark. The DNA content was assessed by a Nexcelom Cellometer (Nexcelom Bioscience Lawrence) and results were analyzed using De Novo FCS Express 4 software.

2.5. Immunophenotyping

Flow cytometry was used to assess the cell surface marker profile of hUC cells. All experiments for FACS analysis were conducted with LP and high passage (HP, >P15) cells. Briefly, the cells were grown to 70% confluency, trypsinized, washed with PBS and pelleted. Cells (10^6) were then stained directly with FITC-conjugated antibodies against: CD34, CD44, CD45, CD90, or APC-conjugated antibodies against: CD29, CD73, CD105 (Becton Dickinson). Cells were stained single or dual labeled and then analyzed on a FACS Canto II (Becton Dickinson) using Diva Software (Beckton Dickinson).

2.6. Colony forming efficiency assay

Cells were seeded at a concentration of 1.6 cells/cm² in a petri dish in triplicate and cultured using CM. After 10–14 days, cells were washed with PBS, fixed in 4% paraformaldehyde for 30 min, stained with 0.1% crystal violet (Thermo Scientific) for 1 h, and then rinsed in tap water. Colonies consisting of a minimal cell number of 50 cells were counted. Data was recorded as total colony number per number of plated cells.

2.7. Lineage differentiation

LP cells were induced to differentiate along chondrogenic, osteogenic, and adipogenic lineages by culturing in specific differentiation media. Chondrogenic differentiation was induced in monolayer and pellet cultures (obtained by centrifugation of 2.5×10^5 cells at 3000 RPM for 10 min) using chondrogenic medium containing 20 ng TGF β 1, 10 ng insulin, 100 nM dexamethasone, and 100 μ M ascorbic acid. After 3 weeks of culture, cells were stained with 1% toluidine blue and Periodic acid–Schiff (PAS) reagent (Thermo Scientific) to detect extracellular matrix produced by chondrogenic derivatives of hUC cells. Osteogenic differentiation was induced by using osteogenic medium containing 0.1 μ M dexamethasone, 10 μ M β -glycerophosphate, and 50 μ M ascorbate-phosphate. After 3 weeks of culture, cells were stained with alizarin red stain (Sigma) and von Kossa stain (Thermo Scientific) to visualize calcium deposition. Adipogenic differentiation was induced using adipogenic medium containing 0.5 μ M isobutyl-methylxanthine, 1 μ M dexamethasone, 10 μ M insulin, and 200 μ M indomethacin. After 3 weeks of culture, cells were stained with Oil Red O (Sigma) to determine the presence of lipid droplets. Cell cultures using CM served as negative controls.

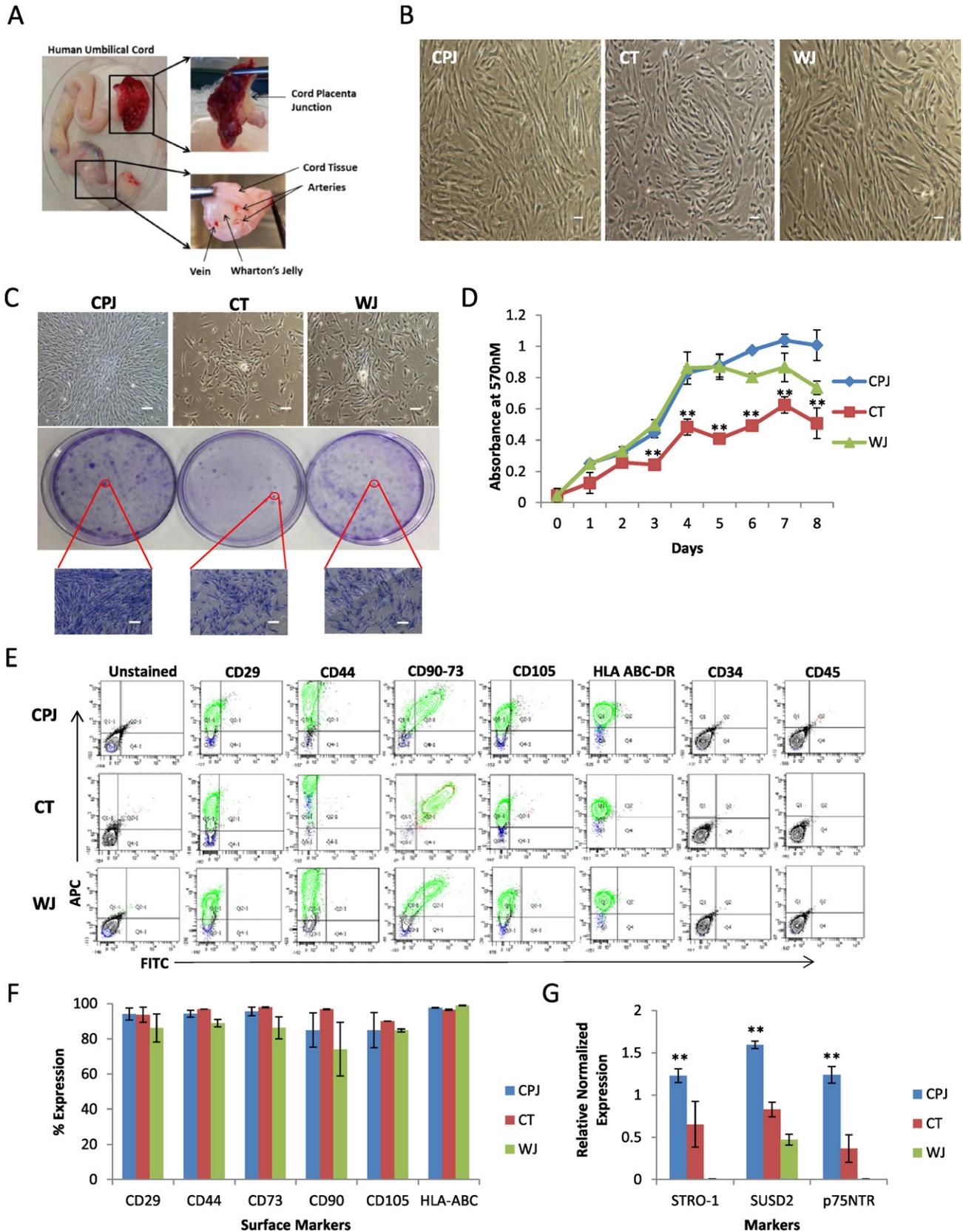
2.8. Sulfated glycosaminoglycans (sGAGs) assay

hUC cell pellets were incubated for 3 weeks in chondrogenic differentiation medium and digested at 55 °C for 16 h with 125 μ g/ml papain (Sigma) in 100 mM sodium phosphate buffer containing 10 mM EDTA, pH 6.5. Cell lysates were vortexed multiple times and cleared by centrifugation. sGAGs were determined by incubating the lysate with dimethylmethylene blue (DMMB) dye in glycine/NaCl solution, pH 3.0 and the complex formed was quantified spectrophotometrically at absorbance 525 nm. Total sGAG content was determined by using the chondroitin sulfate as a standard.

2.9. Immunocytochemical staining

Cells were grown on coverslips, and fixed with 4% paraformaldehyde (USB Products) for 10 min at room temperature. They were then permeabilized with 0.5% Triton X-100 (Sigma) and blocked in 2% bovine serum albumin (Sigma) in PBS for 1 h. Then the cells were subjected

to primary antibody at 1:100 dilutions at 4 °C overnight followed by incubation with secondary antibody at 1:200 dilutions at 37 °C for 1 h. Fluorescently labeled cells were counterstained with DAPI at 1:200 dilutions for 5 min at room temperature and mounted on to the slide. Fluorescent images were captured using a confocal microscope (NIKON Instruments Inc.). The primary and secondary antibodies used are listed



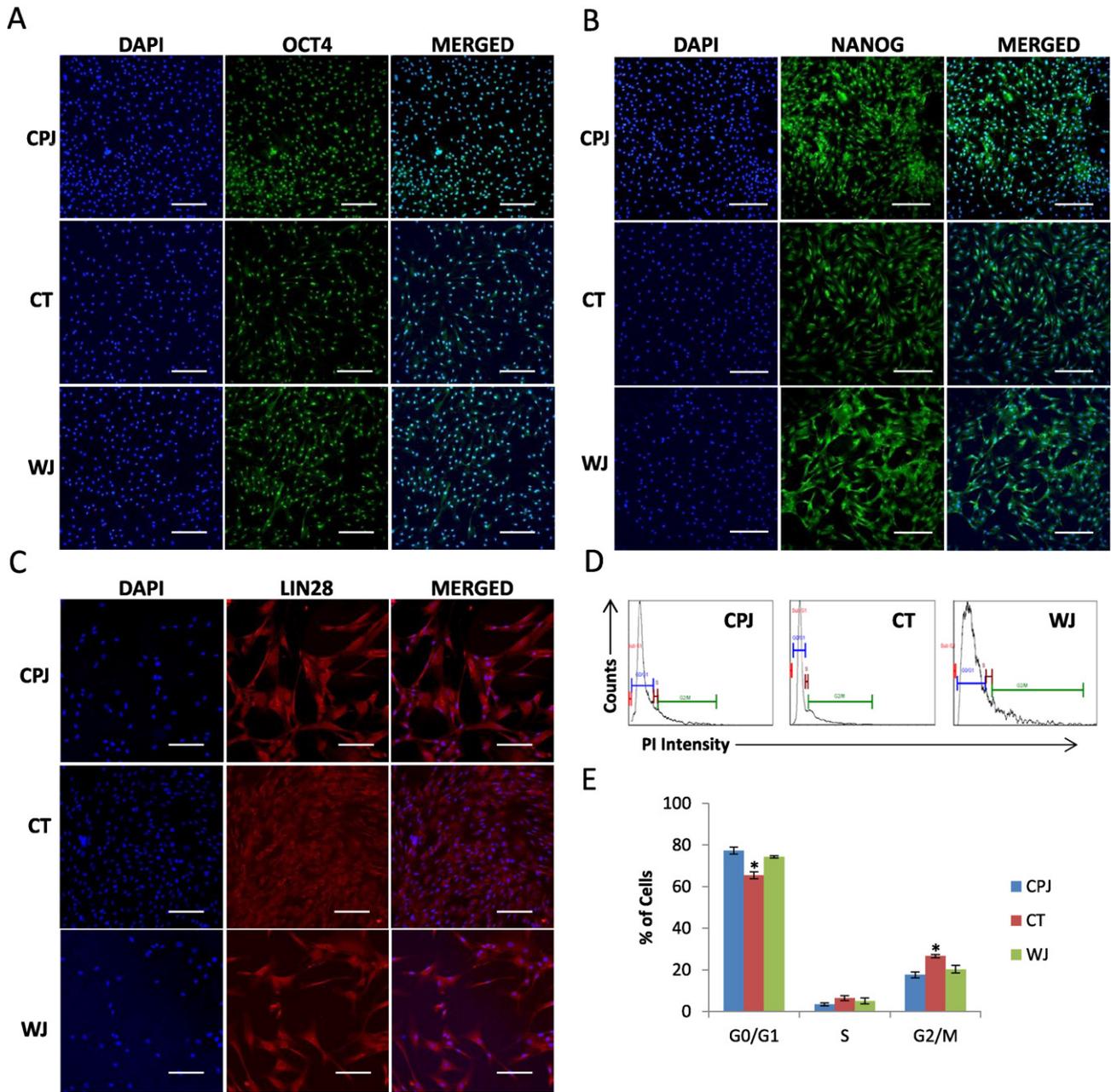


Fig. 2. Expression of pluripotency markers and cell cycle analysis of hUC cells. A–C: Protein expression of OCT4, NANOG, and LIN28, respectively, in hUC cells as determined by immunocytochemical staining. Scale bars represent 100 μ m (magnification: 10 \times). D and E: Histogram and bar graph representing various phases of cell cycle of CPJ-, CT-, and WJ-hUC cells, respectively (* $p \leq 0.05$). CT-hUC cells had a significantly lower percentage of cells in G0/G1 phase as compared to CPJ- and WJ-hUC cells. All the experiments were performed in triplicate.

in Table S2. Non-immune immunoglobulins of the same isotype as the primary antibody were used as negative controls.

2.10. Microarray analysis

LP and HP hUC cells were cultured using CM and harvested at 70% confluency. Cells were washed with PBS and pellets were stored at

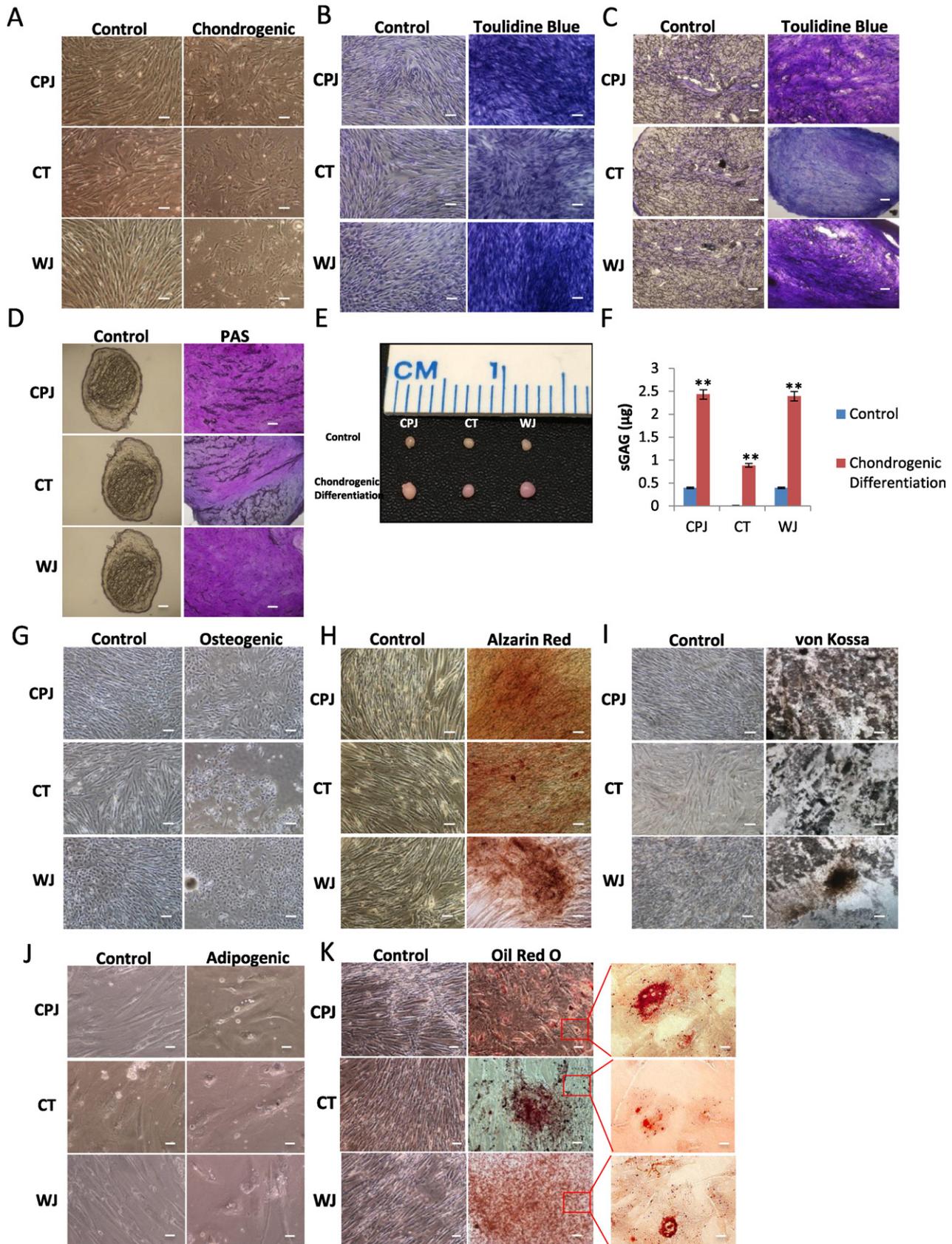
– 80 $^{\circ}$ C. RNA was isolated from frozen cell pellets using the E.Z.N.A. Total RNA Kit I (Omega). Following the manufacturer’s protocol, RNA was purified using spin cartridge technology, quantified (Nanodrop 8000, Thermo Scientific), and then stored at – 80 $^{\circ}$ C.

RNA was then amplified and labeled using the GeneChip™ WT PLUS Reagent Kit (Affymetrix), which enables amplification and target preparation for whole transcriptome expression analysis. Amplification was

Fig. 1. Derivation and characterization of potential stem/progenitor cells from human umbilical cord. A: Anatomical sites of the hUC; arteries, vein, cord placenta junction (CPJ), cord tissue (CT) and Wharton’s jelly (WJ). B: Phase contrast images of cells derived from CPJ, CT, and WJ displaying homogeneous fibroblastoid morphology. C: Phase contrast images of cells derived from CPJ, CT, and WJ displaying clonal growth of cells when replated. Photomicrographs are of LP (low passage) hUC cells stained with crystal violet displaying colony forming capacity. Scale bars represent 100 μ m (magnification: 4 \times). D: Proliferation rate of stem cells as determined by using MTT assay. (** $p \leq 0.01$). The initial proliferation rate of cells derived from CPJ and WJ were similar but higher than cells derived from CT. E and F: Expression and graphical representation of data depicting percentages of cells expressing selected cell surface markers, respectively, as determined by flow cytometry. Cells derived from all three sites (CPJ, CT and WJ), were positive for CD29, CD44, CD73, CD90, CD105 and HLA-ABC, and negative for HSC markers, CD34 and CD45 as well as HLA-DR. G: Expression of STRO-1, SUSD2, p75NTR in cells derived from CPJ, CT, and WJ. Gene expression was normalized to GAPDH and ACTIN and error bars represent the standard deviations of the triplicate measurements.

performed with 500 ng of total RNA input following procedures described in the WT PLUS Reagent Kit user manual. The amplified cDNA was quantified, fragmented, and labeled in preparation for hybridization to GeneChip™ Human Transcriptome 2.0 Arrays (Affymetrix)

using 5.5 µg of ss-cDNA product following protocols outlined in the aforementioned user manual. Washing, staining (GeneChip® Fluidics Station 450, Affymetrix) and scanning (GeneChip® Scanner 3000, Affymetrix) were performed following protocols outlined in the



GeneChip™ Expression Wash, Stain, and Scan User Manual for Cartridge Arrays. Analysis was conducted in Affymetrix Transcriptome Analysis Console (version 1.0.0.234) and sub-network enrichment analysis (SNEA), differential gene expression was determined by One-Way Between-Subject ANOVA (Unpaired), with p-value <0.05 and a 2-fold cutoff and $p \leq 0.05$ and 1.5-fold cutoff respectively. Hierarchical clustering was carried out using Partek® Genomics Suite 6.6 software (version 6.14.0923, Partek Inc.). Hierarchical clustering analysis was performed using Euclidean distance as similarity measure and average linkage for the agglomerative method. The accession numbers for the microarray data reported in this paper are NCBI GEO: GSE72597 and GSE76295.

2.11. Quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) analysis

Cells were grown under appropriate conditions, harvested and used to isolate total cellular mRNA using the GeneJET RNA purification Kit (Thermo Scientific), following the manufacturer's instructions. Total RNA was purified by incubation with DNase at 37 °C for 30 min by using a thermocycler (Bio-Rad). cDNA was synthesized by using iScript kit (Bio-Rad). qRT-PCR was performed by using Sso-Advanced Universal SYBR Green Supermix Kit (Bio-Rad) on CFX96 Real-Time System (Bio-Rad). A 10 µl reaction was used including 5 µl of Syber green, 3 µl of distilled H₂O, 0.5 µl of forward primer, 0.5 µl of reverse primer, and 1 µl of 1:10 diluted cDNA. Each reaction was subjected to the following conditions: 98 °C for 10 min, followed by 44 cycles of 98 °C for 30 s, 60 °C for 20 s, and 72 °C for 30 s in 96-well optical reaction plates (Bio-Rad). Reference genes, GAPDH and ACTIN, were used to normalize the amplification of the target genes. Each qRT-PCR analysis was performed in triplicate. Primer sequences are listed in Table S3.

2.12. Statistical analysis

Data are presented as the mean ± SEM. Results with a p-value ≤0.05 were considered statistically significant. All analyses were performed using SPSS version 11.5 (SPSS Inc.).

3. Results

3.1. Derivation and characterization of hUC cells

In our preliminary studies with whole hUC, we isolated a heterogeneous population of colony forming cells. In fact, some reports had indicated isolation of colony forming cells from CT and WJ (Ding et al., 2015). This prompted us to dissect the hUC into identifiable anatomical sites as depicted in Fig. 1A, and to isolate adherent cells from each part separately. A significant outgrowth of adherent cells was observed within 3–4 days and 7–10 days in explants of CPJ and both CT and WJ, respectively. Our attempts to isolate cells from the tissues enzymatically had low yield and lacked reproducibility. Initially, isolated outgrowth cells, irrespective of the anatomical sites, appeared to be fibroblastoid and expanded colonially when replated as shown in Fig. 1B and C, respectively. In addition, the results of Colony Forming Efficiency (CFE) assays presented in Fig. 1C revealed variation in the hUC cells resulting from passaging. CFE was determined to be 92, 59, and 80 colonies per

100 LP CPJ-, CT-, and WJ-hUC cells, respectively. LP CPJ- and WJ-hUC cells displayed higher colony forming capacity than CT-hUC cells. The results of a proliferation assay reported in Fig. 1D demonstrate that CPJ-hUC cells had a significantly higher growth rate when compared with CT- and WJ-hUC cells.

Because the isolated cells from three sites of the hUC were adherent, and were able to form colonies, we wondered if they were similar to stem/progenitor cells found in other connective tissues. It has been suggested that cells loosely defined as “MSCs” express certain cell surface markers (Dominici et al., 2006; Robey et al., 2014), however, these markers are not specific (Dominici et al., 2006; Robey et al., 2014). Nevertheless, we subjected the isolated cells to immunophenotyping using flow cytometric analysis. The results reported in Fig. 1E and F show that cells derived from all three sources expressed CD29, CD44, CD73, CD90, and CD105, but not the hematopoietic specific markers, CD34 and CD45. These cells were also found to be positive for major histocompatibility class I, HLA-ABC and negative for class II, HLA-DR (also see Table S1). Isolated P0 cells had 71–98% of cells expressing these markers; however, homogenous cell populations (95–99%) expressing these markers was achieved upon further passaging (P1–P2). In addition, hUC cells also expressed markers such as STRO-1, SUSD2 and p75NTR as shown in Fig. 1G. Again, expression of these markers was highest in CPJ-hUC cells. Interestingly, these markers have been recently used along with others to identify BMSCs (Lin et al., 2011; Sivasubramaniyan et al., 2013). Further immunocytochemical analysis of the isolated cells revealed that they also expressed pluripotency genes, OCT4, NANOG, and LIN28 as shown in Fig. 2A–C. Protein expression of these genes was higher in both CPJ- and WJ-hUC cells than CT-hUC cells. However, levels of these markers were much lower than in bona fide pluripotent ESCs (see Fig. S1).

The growth potential of the cells varied with the site from which they originated, which prompted us to investigate the cell cycle of these cells. A comparative analysis of the cell cycle of the cells derived from all three sites is presented in Fig. 2D and E. Evidently; CPJ- and WJ-hUC cells had similar G₀/G₁ and G₂/M phases with a range of 75–80% and 18–20% cells, respectively. Whereas, in case of CT-hUC cells, G₀/G₁ phase had 10% lower but G₂/M phase had 10% higher cells. These results were consistent with the proliferation rate of the cells from the three sites as stated above.

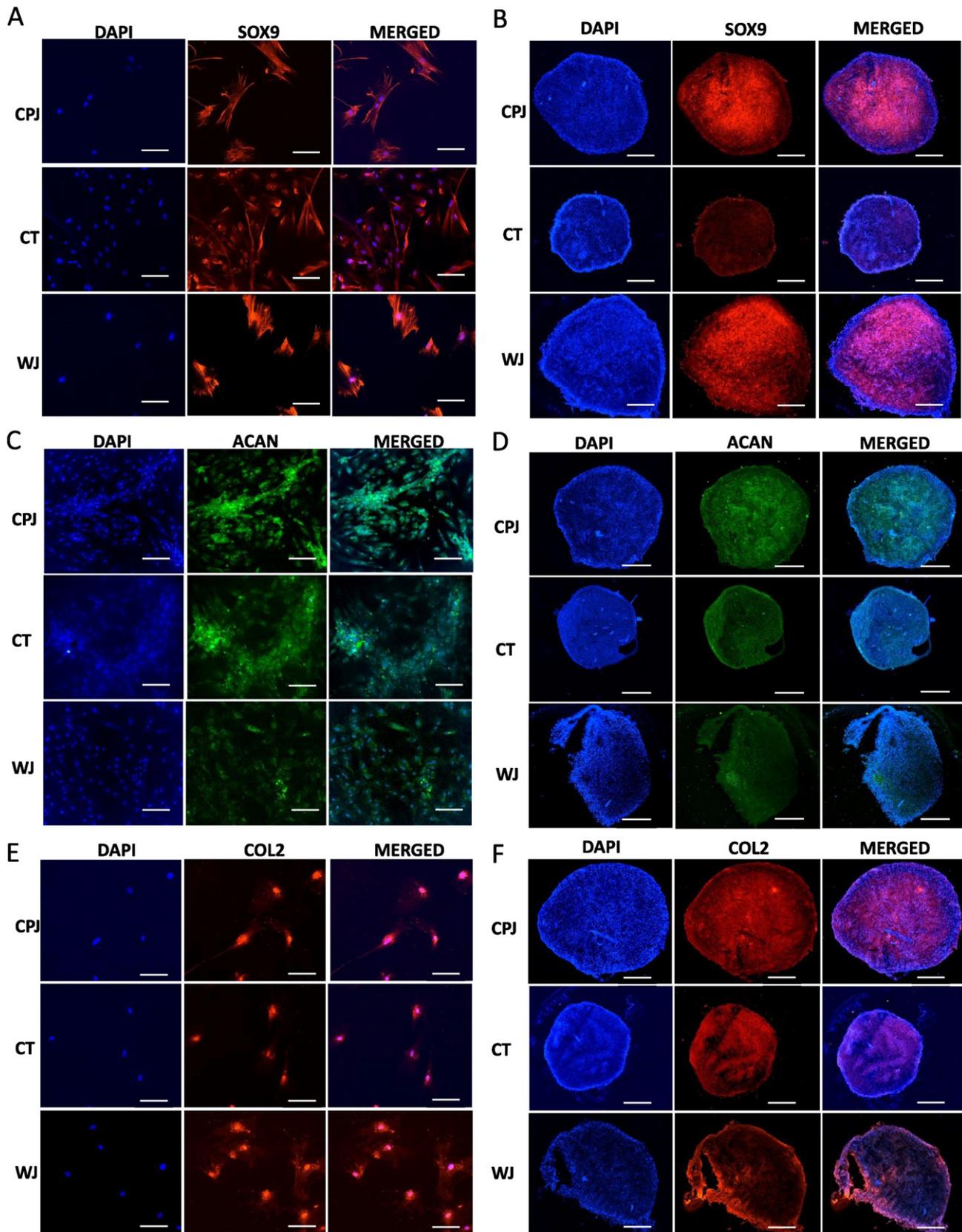
3.2. Multi-lineage differentiation potential of hUC cells

Based on their cell surface similarity to BMSCs, and their peri-natal nature, the ability of hUC cells to differentiate towards mesenchymal lineages (chondrogenic, osteogenic, and adipogenic cells) in vitro was examined by culturing them in selective differentiation media. Monolayer cells incubated in chondrogenic medium for 3 weeks displayed altered morphology as shown in Fig. 3A. Staining of the chondrogenic derivatives of hUC cells with toluidine blue as depicted in Fig. 3B as well as alcian blue (see Fig. S2) demonstrated production of GAG suggesting that cells from all three sites differentiated into chondrogenic lineage. Furthermore, histological sections of the pellet cultures also stained positive for toluidine blue and PAS staining as shown in Fig. 3C and D indicating that the differentiated derivatives produced proteoglycans and glycoproteins. In addition, the size of the pellets increased

Fig. 3. Multi-lineage differentiation of hUC cells in vitro. A–F: Chondrogenic differentiation of CPJ-, CT- and WJ-hUC cells. The cells were cultured as monolayers or as pellets in chondrogenic medium for 3 weeks. The cell morphology as determined by phase contrast microscopy and toluidine blue staining of monolayer chondrogenic derivatives of hUC cells (A and B, respectively). Histological sections of pellet cultures of chondrogenic derivatives stained with toluidine blue and PAS displaying presence of glycosaminoglycan (GAG) and glycoproteins (C and D, respectively). Photographic representation of pellet size of hUC cells after culture in chondrogenic medium (E). Sulfated GAG (sGAG) content of pellets of hUC cells cultured in chondrogenic medium as determined by DMMB assay and GAG contents were normalized to chondroitin sulfate as positive control (F). Pellet size and sGAG content of CPJ- and WJ-hUC cells were significantly greater than CT-hUC cells. G–I: Osteogenic differentiation of CPJ-, CT- and WJ-hUC cells grown for 3 weeks in the osteogenic medium. The cell morphology as determined by phase contrast microscopy, alizarin red and von Kossa staining of osteogenic derivatives of hUC cells (G–I, respectively). Significantly higher staining of osteogenic derivatives compared with the control hUC cells suggests bone mineralization by production of calcium deposits. J–K: Adipogenic differentiation of CPJ-, CT- and WJ-hUC cells grown for 3 weeks in the adipogenic medium. The cell morphology as determined by phase contrast microscopy, and Oil Red O staining of adipogenic derivatives of hUC cells (J–K, respectively). Formation of lipid droplets is an indicative of adipogenic differentiation. Scale bars represent 100 µm (magnification: 4×).

upon incubation in chondrogenic differentiation medium as shown in Fig. 3E indicating extracellular matrix production, providing further evidence of differentiation into chondrogenic lineage. The quantification of sGAGs shown in Fig. 3F indicates 2-fold increase in CPJ- and

WJ-hUC cells derivatives relative to the control, compared to CT-hUC cells. The variation in the pellet size and GAG content of cells from the three sites incubated in chondrogenic medium could be attributed to their differential chondrogenic potential, with CPJ- and



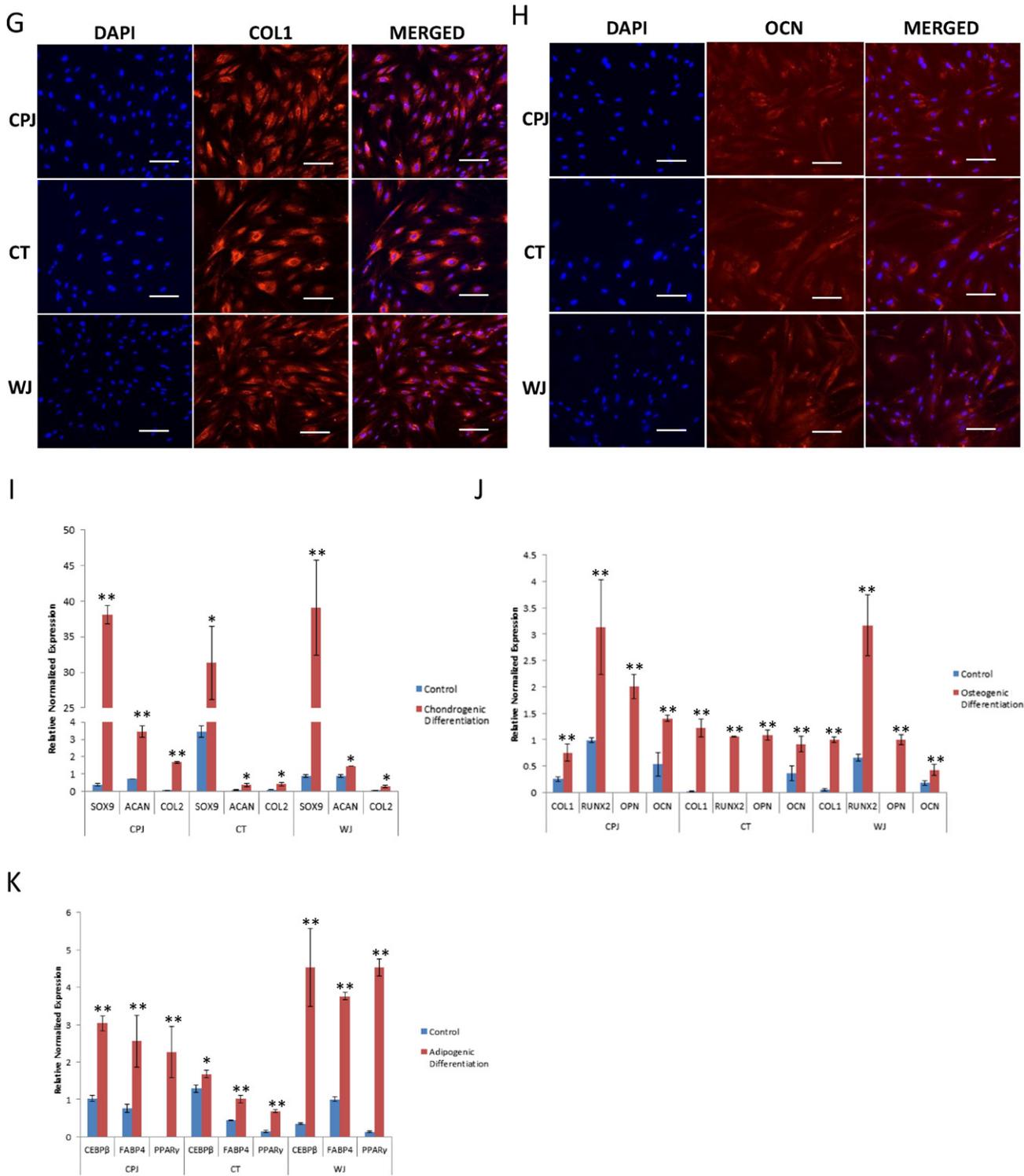


Fig. 4. Expression of cell specific proteins and genes in differentiated derivatives of hUC cells. The cells were cultured in respective differentiation media for 3 weeks and subjected to analysis. A-F: Protein expression of SOX9, aggrecan (ACAN) and collagen 2 (COL 2), in chondrogenic derivatives of CPJ-, CT- and WJ-hUC cells in monolayer cultures (A–C, respectively) and pellet cultures (D–F, respectively). G–H: Protein expression of collagen 1 (COL 1) and osteocalcin (OCN), in osteogenic derivatives of CPJ-, CT- and WJ-hUC cells, respectively, as determined by immunocytochemical analysis. Scale bars represent 100 μm (magnification: 10×). I–K: Transcriptional levels of selected genes, for chondrogenic (SOX9, ACAN, and COL2), osteogenic (COL1, RUNX2, OPN, and OCN) and adipogenic (CEBPβ, FABP4, and PPARγ) lineages, as determined by qRT-PCR analysis (**p ≤ 0.01 and *p ≤ 0.05). Gene expression was normalized to GAPDH and ACTIN and error bars represent the standard deviations of the triplicate measurements.

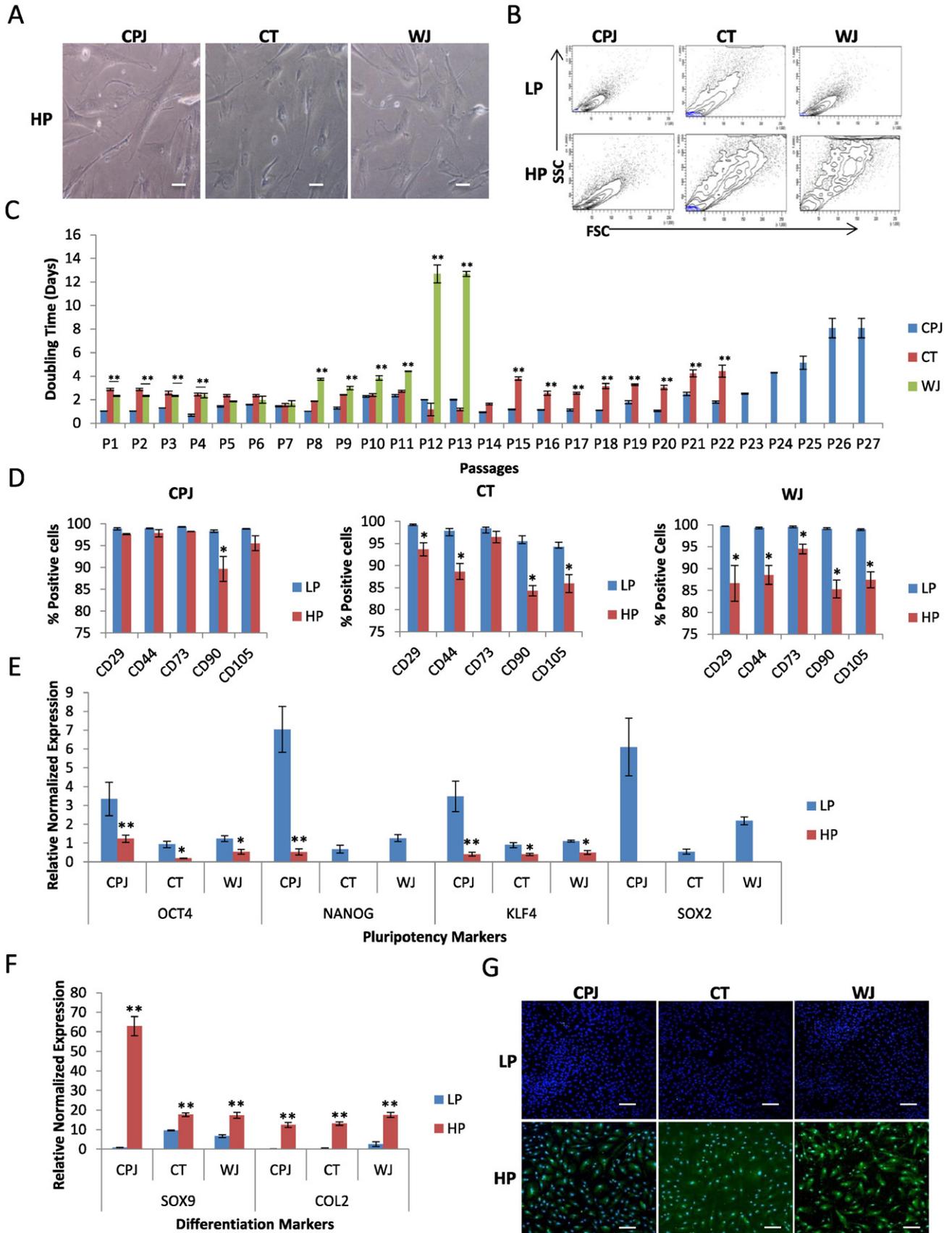
WJ-hUC cells displaying better chondrogenic differentiation than CT-hUC cells.

When hUC cells were cultured in osteogenic differentiation medium for 3 weeks, they displayed a cuboidal morphology as depicted in Fig.

3G. The osteogenic derivatives from all three sites also stained positive with alizarin red and von Kossa as shown in Fig. 3H and I, respectively, indicating the presence of calcium phosphate deposits. Likewise, when hUC cells were induced towards the adipogenic lineage, they produced

lipid droplets that were positively stained with Oil Red O as presented in Fig. 3J and K, respectively. Furthermore, we investigated the expression of cell-specific proteins in the differentiated cells. The results of

immunocytostaining of chondrogenic derivatives of in hUC cells both from monolayer and pellet culture depicted in Fig. 4A–C and D–F, respectively, demonstrated the expression of chondrogenic markers,



SOX9, aggrecan (ACAN) and collagen 2 (COL2). Similarly, immunocytochemical staining of osteogenic derivatives of hUC cells presented in Fig. 4G and H, respectively, showed expression of characteristic osteogenic markers, collagen 1 (COL1) and osteocalcin (OCN). qRT-PCR results of transcriptional analysis presented in Fig. 4I show increased expression of chondrogenic genes, SOX9, ACAN and COL2 in the differentiated derivatives. Highest expression of these markers was observed in chondrogenic derivatives of CPJ-hUC cells. Likewise transcriptional analysis of osteogenic derivatives of hUC cells in Fig. 4J revealed increased expression of osteogenic genes such as COL1, RUNX2, osteopontin (OPN) and osteocalcin (OCN). Levels of expression of the osteogenic marker, RUNX2, were greater in osteogenic derivatives of both CPJ- and WJ-hUC cells than CT-hUC cells. Similar analysis of adipogenic derivatives illustrated in Fig. 4K shows an increased expression of adipogenic genes such as, CCAAT/enhancer-binding protein beta (CEBP β), fatty acid-binding protein (FABP4), and peroxisome proliferator-activated receptor gamma (PPAR γ) compared to control. Highest expression of these markers was observed in derivatives of WJ-hUC cells than CPJ- and CT-hUC cells. These in vitro results supported the notion that the hUC-derived cells may have multi-lineage differentiation potential.

3.3. Effect of passaging on the hUC cells

The growth rate of isolated hUC cells progressively decreased and the cell size increased upon passaging as shown in Fig. 5A and B. These changes were more pronounced in CT- and WJ-hUC cells. The CFE also markedly declined in all HP hUC cells (see Fig. S3). A significant drop in CFE can be seen in both CT- and WJ-hUC cells at P10 and in CPJ-hUC cells at P20. The population doubling (PD) time as shown in Fig. 5C was also affected by passaging. hUC cells were cultured until growth ceased and PD time was measured for every passage, CPJ-hUC cells showed significantly shorter doubling time and greater number of PDs as compared to CT- and WJ-hUC cells. WJ-hUC cells had the shortest culture time as cell growth arrested at P13. However, CT-hUC cells displayed a longer PD time, but proliferation did not stop until P22. The shortest PD time and longest culture periods were observed in the case of CPJ-hUC cells, as proliferation stopped at P27. We also found that passaging resulted in the loss of cell surface markers. FACs analysis presented in Fig. 5D shows the overall loss of cell surface markers in HP hUC cells. While the loss of CD90 was more pronounced than any other marker in HP hUC cells from all three sites, other markers were less affected in CPJ-hUC cells.

Further analysis of the hUC cells revealed that passaging also caused rapid loss of pluripotency gene expression. A comparison of the expression of OCT4, SOX2, NANOG and KLF4 in both LP and HP hUC cells as determined by qRT-PCR is presented in Fig. 5E. Although loss of all tested markers was significant, SOX2 was completely lost in HP hUC cells from all three sites and NANOG was lost in both HP CT- and WJ-hUC cells. It should be noted, however, SOX2 and NANOG were 4- and 6-fold higher in LP CPJ-hUC cells compared to LP CT- and WJ-hUC cells, respectively. Additionally, the results shown in Fig. 5F and G show expression of markers indicative of the chondrogenic lineage without the cells being cultured in chondrogenic medium as evidenced by the increased transcript levels of SOX9 and COL2, and protein expression of COL2, respectively. All together these results indicate that CPJ-hUC cells had higher growth and differentiation potential than CT- and WJ-hUC cells.

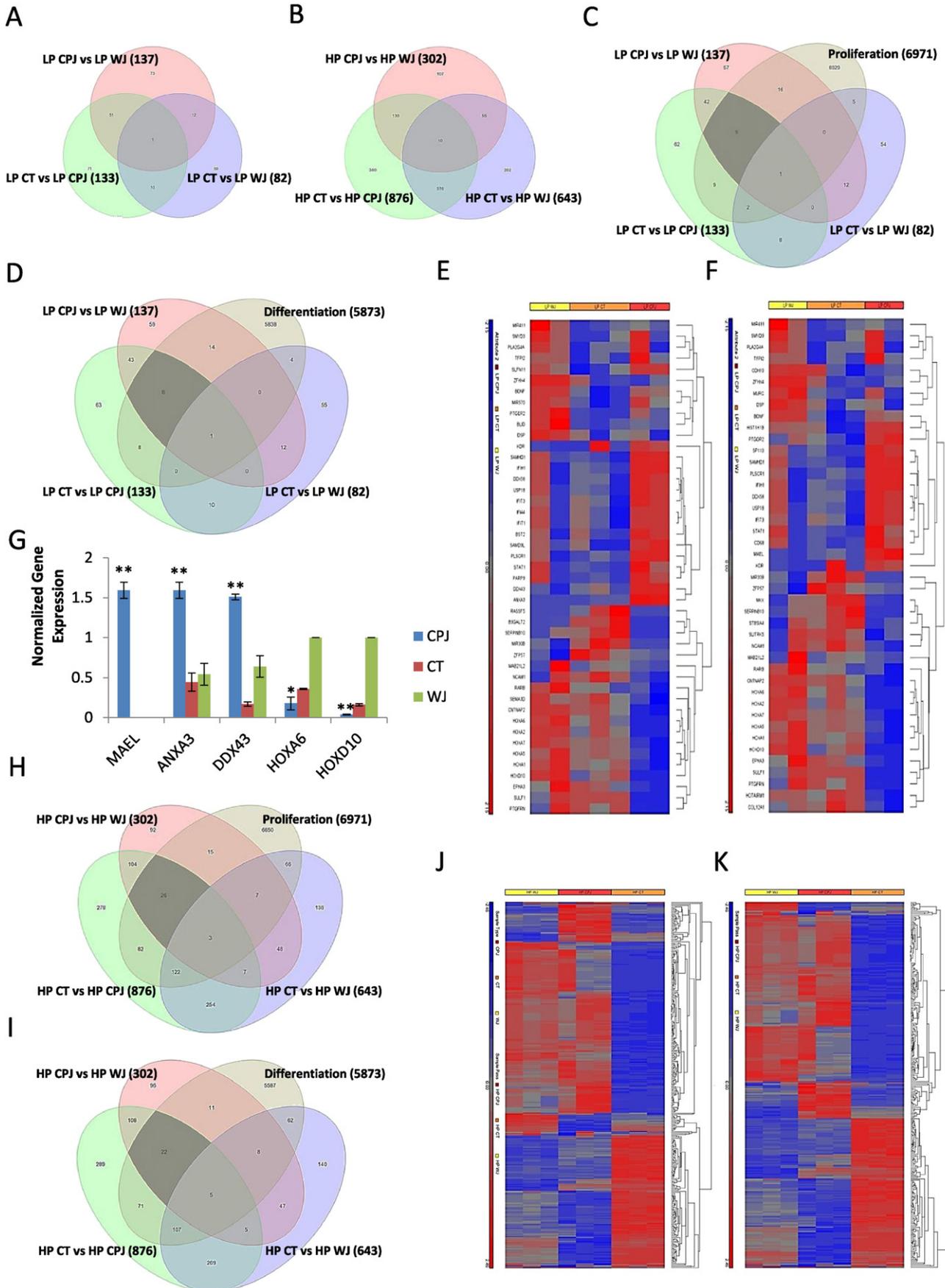
3.4. Microarray analysis of hUC cells

Since hUC cells derived from the three different sites displayed significant differences, we probed the global transcriptome of LP and HP hUC cells. The Venn diagram of LP hUC cells transcriptome analysis depicted in Fig. 6A shows that overall, 352 genes were differentially expressed among hUC cells isolated from three sites of hUC. Out of these, 133 genes were differentially expressed between hUC cells from CPJ vs. CT (90 upregulated and 43 downregulated), 137 genes were differentially expressed between hUC cells from CPJ vs. WJ (90 upregulated and 47 downregulated), and 82 genes were differentially expressed between hUC cells from CT vs. WJ (27 upregulated and 55 downregulated). There was only one overlapping gene, HOXD10, which was common to all hUC cells.

When a similar global transcriptome analysis was performed with HP hUC cells, unlike LP hUC cells, significantly large numbers of genes were found to be differentially expressed among CPJ-, CT-, and WJ-hUC cells. The Venn diagram of HP hUC cells transcriptome analysis depicted in Fig. 6B shows that overall 1821 genes were differentially expressed among cells isolated from three sites of hUC. Out of these, 876 genes were differentially expressed between hUC cells from CPJ vs. CT (434 upregulated and 442 downregulated), 302 genes were differentially expressed between hUC cells from CPJ vs. WJ (211 upregulated and 91 downregulated), and 643 genes were differentially expressed between hUC cells from CT vs. WJ (319 upregulated and 324 downregulated). Overall, in the global transcriptome analyses of HP hUC cells, there were ten genes that were common in all the differentially expressed genes found in hUC cells from three sites of the hUC. Out of these ten genes, five genes were coding for FMOD, SULF1, COL12A1, STMN2 and MAEL, and the remaining five were long-non-coding RNA genes (lncRNA). Interestingly, among the five coding genes, three genes (FMOD, COL12A1, and SULF1) belong to a family of proteoglycans and an enzyme associated with extracellular matrix production, and is expressed in chondrogenic lineage. Their expression was highest in CT-hUC cells and lowest in CPJ-hUC cells. Expression of STMN2, involved in neuronal growth associated protein, was found to be highest in WJ-hUC cells and lowest in CPJ-hUC cells. Higher expression of these genes could be attributed to the lower rate of proliferation in HP CT- and WJ-hUC cells. MAEL was the only gene that was differentially expressed in both LP and HP hUC cells. In both the cases, its expression was highest in CPJ-hUC cells and lowest in WJ-hUC cells. However, the significance of these differences remains to be investigated.

Because hUC cells from different sites of hUC had different properties, particularly relating to their proliferation and differentiation, we further analyzed the microarray data to decipher the expression of genes involved in these functions. Results of LP hUC cells analysis are illustrated in Venn diagrams (Fig. 6C and D) and heatmaps (Fig. 6E and F) for proliferation and differentiation gene categories, respectively. The expanded heatmaps for detailed gene names are presented in Figs. S5 and S6. It is clear that out of all the proliferation genes ($n = 6971$), 45 genes were differentially expressed, which overlapped in all LP hUC cells. However, 9 genes were not differentially expressed in hUC cells from CT vs. WJ. Likewise, among the differentiation genes ($n = 5873$), 43 genes were expressed differentially and overlapped in all LP hUC cells except 8 genes that were not differentially expressed in hUC cells from CT vs. WJ. Interestingly, 7 of these genes (SULF1,

Fig. 5. Comparison of morphology, proliferation, and expression of selected markers at low and high passages of CPJ-, CT-, and WJ-hUC cells. A: Phase contrast images of HP hUC cells displaying increased cell size compared with LP hUC cells shown in Fig. 1. B: Cell size analysis of LP and HP hUC cells by using flow cytometry. C: Doubling time of hUC cells increased upon passaging. CPJ-hUC cells showed a higher number of population doublings than CT- and WJ-hUC cells. (** $p \leq 0.01$). D: Comparison of expression of selected hUC cells markers in LP and HP hUC cells. (* $p \leq 0.05$). E and F: Transcription analysis of pluripotency genes, OCT4, NANOG, KLF4 and SOX2 and chondrogenic genes, SOX9 and COL2 in hUC cells, as determined by qRT-PCR. (** $p \leq 0.01$ and * $p \leq 0.05$). Gene expression was normalized to GAPDH and ACTIN and error bars represent the standard deviations of the triplicate measurements. G: Protein expression of COL2 in both LP and HP hUC cells as determined by immunocytochemical staining. Scale bars represent 100 μm (magnification: 10 \times). CPJ-hUC cells had higher expression of pluripotency markers. Expression of COL2 was increased in HP hUC cells compared to LP hUC cells.



BCHE, HAOTAIRM1, HOXA5, HOXA6, PPTN3, and SHOX2) were also found in the category of proliferation genes. Therefore, in both the proliferation and differentiation categories, there were a total of 11 genes that were not differentially expressed in CT vs. WJ. 4 of these 11 genes (ANXA3, SHOX2 and DDX43 and MAEL) were distinctly expressed higher, and 7 genes (SULF1, BCHE, SEMA3D, HOXA5, HOXA6, HOXD10, PTPN3 and HOTAIRM1) were distinctly expressed lower in LP CPJ-hUC cells. These results indicate that these 11 genes might play a role in the different properties of CPJ-hUC cells. Differential expression of these genes in hUC cells was confirmed by qRT-PCR (Fig. 6G). However, it remains to be determined whether the observed differences in hUC cells properties from three sites of hUC can be attributed to the differential expression of these genes.

A similar analysis performed for the HP hUC cells is illustrated in Fig. 6J and K, which shows that out of all the proliferation genes, 349 differentially expressed genes overlapped in all HP hUC cells except 26 genes that were not differentially expressed in hUC cells from CT vs. WJ. Likewise, out of all the differentiation genes, 302 genes were differentially expressed which overlapped in all hUC cells, but 22 genes were not differentially expressed in hUC cells from CT vs. WJ. Taken together, there were 14 genes (ENPP2, ITGA2, GPR158, AFAP1-AS1, SHOX2, ANPEP, PDE5A, NR5A2, ARHGDB, S100 A4, ABCG2, SESN3, ASPN, and RNF17) that revealed higher expression in CPJ-hUC cells, and 5 genes (HOXA6, RTN1, CPPED1, HOXA7 and MGST1) that were distinctly expressed higher in WJ-hUC cells, which might play a role in the cessation of cell proliferation. Interestingly, two differentiation genes (HOXA6 and HOXD10) that were downregulated in LP CPJ-hUC cells were also found among the 13 downregulated genes in HP CPJ-hUC cells. However only one of the two proliferation genes (ANXA3) that was upregulated in LP CPJ-hUC cells was among the upregulated 18 genes in HP CPJ-hUC cells. Only one gene (MEAL) was upregulated in both LP and HP CPJ-hUC cells. The expanded heatmaps for detailed gene names are presented in Figs. S9 and S10.

Since hUC cells were affected by passaging, it was of interest to compare the gene expression between the HP vs. LP hUC cells isolated from all three sites of the hUC. The results shown in Fig. 7A indicate that 2174 genes were differentially expressed between HP vs. LP hUC cells from CPJ (1287 upregulated and 887 downregulated), 2299 genes were differentially expressed between HP vs. LP hUC cells from CT (1637 upregulated and 662 downregulated), and 1910 genes differentially expressed were found between LP vs HP hUC cells from WJ (1260 upregulated and 650 downregulated). A relatively large number of genes, 942, were found to overlap. Therefore, we focused on genes involved only in cell proliferation and differentiation. The results depicted in Fig. 7B–E shows that there were 482 and 370 proliferation and differentiation genes, respectively, which were differentially expressed. The expanded heatmaps for detailed gene names are presented in Figs. S8 and S9. 164 genes were upregulated and 318 genes were downregulated within HP vs. LP hUC cells from CPJ. In HP vs. LP hUC cells from CT, 169 genes were upregulated and 313 genes were downregulated. In HP vs. LP hUC cells from WJ, 160 genes were upregulated and 322 genes were downregulated. Among the differentially expressed genes between HP vs. LP hUC cells from CPJ, 126 genes were upregulated and 244 genes were downregulated. In HP vs. LP hUC cells from CT, 133 genes were upregulated and 237 genes downregulated. In HP vs. LP hUC cells from WJ, 115 genes were upregulated and 367 genes were downregulated. Among the cell proliferation genes in HP vs. LP

hUC cells, 17–33 and 2–3 genes were differentially expressed by >5 fold and >10 fold, respectively. Genes showing greater than 10 fold up-regulation were SLPI and RSAD2 in CPJ-hUC cells, ADAMTS5 in CT-hUC cells, and IFIT2 and RSAD2 in WJ-hUC cells. In contrast, 10 fold downregulated genes were KIF20A and MKI67 in CT-hUC cells and KAL1 in WJ-hUC cells. Similarly, >5 fold differentially expressed genes varied from 10 to 24 in HP vs. LP hUC cells of all three sites. A number of genes involved in category of differentiation were >10 fold differentially expressed in hUC cells including SLPI (upregulated) and MMP1 (downregulated) in HP CPJ-hUC cells, whereas ADAMTS5 was upregulated and MKI67 was downregulated in HP CT-hUC cells, and RSAD2 was upregulated and KAL1 was downregulated in HP WJ-hUC cells.

When the proliferation genes that were highly expressed in LP hUC cells were followed for their expression in HP hUC cells, 8 genes (EPHA3, KDR, NCAM1, PTGFRN, PTGER2, SEMA3D, SULF1, and TFPI2), 5 genes (DSP, EPHA3, NCAM1, PTGFRN, SEMA3D, and SULF1), and 4 genes (DSP, KDR, PTERG2, and TFPI2) were found to be downregulated in HP CPJ-hUC cells, CT-hUC cells, and WJ-hUC cells, respectively. Three genes (KDR, PTERG2, and TFPI2) were found to display similar expression pattern in hUC cells from CPJ and WJ; however, their contributions to the variation in properties of the hUC cells from these two sites remain to be established.

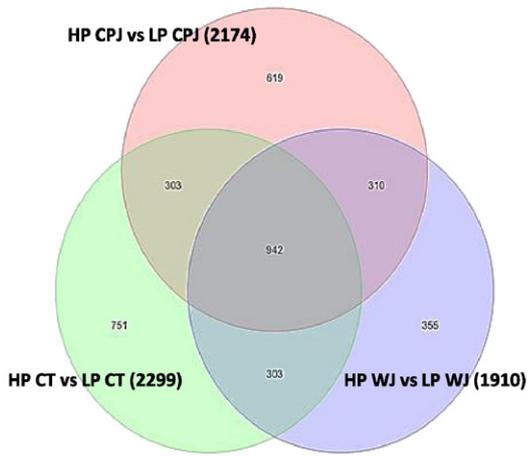
Similarly, the category of differentiation genes that had low expression in LP hUC cells and were upregulated in HP hUC cells included 6 genes (IFIH1, KDR, MAEL, SAMHD1, SP110, and STAT1), 2 genes (CDH10 and PTGFRN), and 13 genes (DDX58, DSP, HOXA5, HOXA6, HOXA7, HOXD10, IFIH1, IFIT3, PLSCR1, SAMHD1, SP110, STAT1 and USP18) in hUC cells from CPJ, CT and WJ, respectively. However, only 3 genes (SAMHD1, SP110, and STAT1) had similar pattern of expression in CPJ- and WJ-hUC cells. Again, their role in determining the variation in properties of the hUC cells from these two sites remains to be determined.

4. Discussion

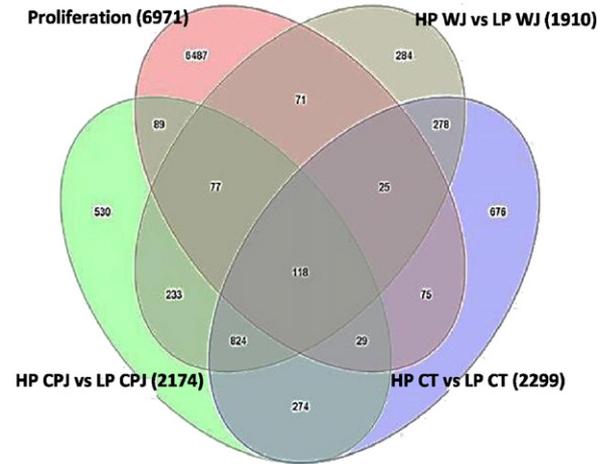
One of the most important areas of interest in ASCs is the source and efficacy of isolation techniques to yield an adequate amount and homogeneous population of cells for tissue engineering and regenerative medicine applications. Bone marrow is a well-recognized source of ASCs (Catacchio et al., 2013) and contains HSCs, and SSCs/BMSCs (Bianco and Robey, 2015; Robey et al., 2014). Isolation of potential stem/progenitor cells from other adult tissues and organs with similar cell surface characteristics as SSCs/BMSCs has also been reported (Cho et al., 2015; Kim et al., 2015; Lee et al., 2013). However, most of these sources require invasive procedures and might have undergone genetic changes due to environmental stresses and the aging process (Adams et al., 2015; Burkhalter et al., 2015; Oh et al., 2014). To address these concerns, putative stem/progenitor cells have been reported to be isolated from noninvasive peri-natal sources such as amniotic fluid and umbilical cord blood (Fei et al., 2013; Odabas et al., 2014; Steigman and Fauza, 2007; Wouters et al., 2007), but the yield is often low (Erices et al., 2000; Goodwin et al., 2001; Rosada et al., 2003). Recently, putative stem/progenitor cells have been also isolated from hUC (Mennan et al., 2013). Stem/progenitor cells from different connective tissues have been reported to display similar cell surface markers, particularly CD29, CD44, CD73, CD90, and CD105 (Dominici et al., 2006). However, other characteristics of putative stem/progenitor cells, such

Fig. 6. Comparative microarray analysis among CPJ-, CT-, and WJ-hUC cells. A–B: Venn diagrams displaying the comparative gene expression in LP and HP hUC cells. C–D and E–F: Venn diagrams and heatmaps displaying the differentially expressed genes involved in cell proliferation and differentiation, respectively, in LP hUC cells. Expanded heatmaps of HP hUC cells are presented in Figs. S5 and 6. G: Expression of selected differentially expressed genes in CPJ-, CT-, and WJ-hUC cells as determined by qRT-PCR. Three genes (MAEL, ANXA3 and DDX43), were expressed higher in CPJ- than in CT- and WJ-hUC cells (** $p \leq 0.01$), whereas, two genes (HOXA6 and HOXD10) had reduced expression in CPJ-hUC cells compared with CT- and WJ-hUC cells (** $p \leq 0.01$). Gene expression was normalized to GAPDH and ACTIN and error bars represent the standard deviations of the triplicate measurements. H–I and J–K: Venn diagrams and heatmaps displaying the differentially expressed genes involved in cell proliferation and differentiation, respectively, in HP hUC cells. Up and downregulated genes are displayed in red and blue, respectively. Expanded heatmaps of HP hUC cells are presented in Figs. S7 and 8.

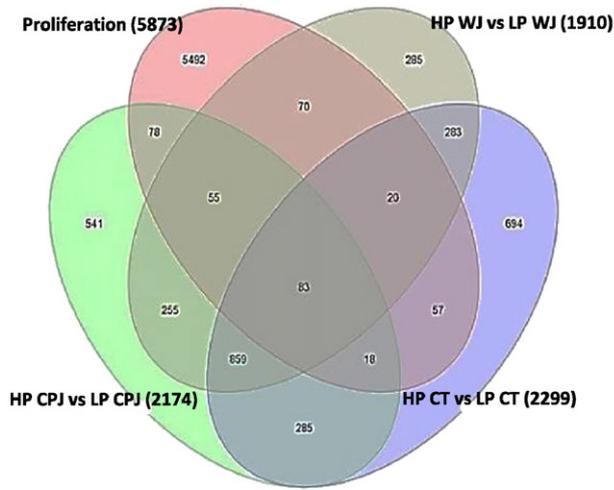
A



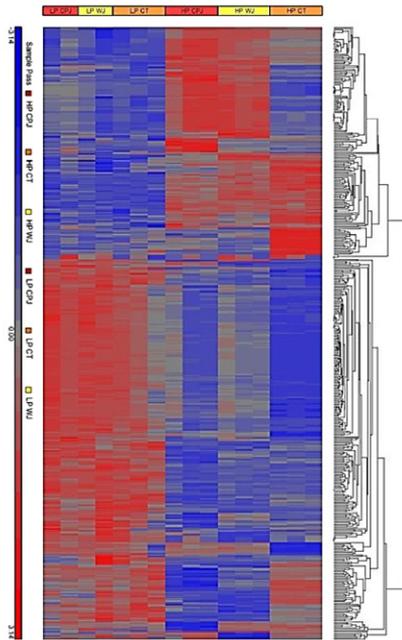
B



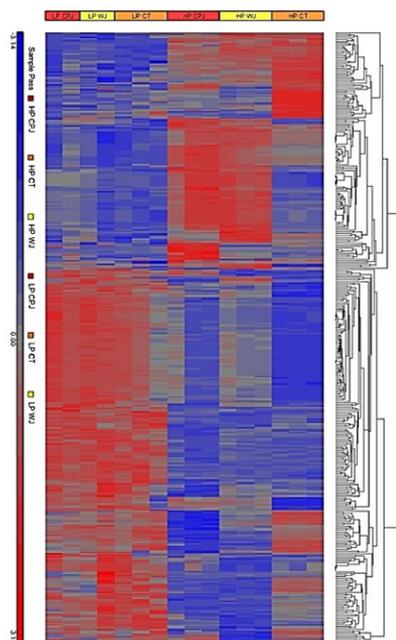
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D



E



as self-renewal and differentiation potential, have not been reported, or vary widely depending upon the tissue source. For example, cells isolated from older individuals or certain tissues have lower self-renewal capacity compared with those isolated from younger individuals and peri-natal sources (Escacena et al., 2015). Apparently, age of the source, and character of the cellular or tissue niche are determining factors for self-renewal and differentiation potential of various stem/progenitor cell populations (Okolicsanyi et al., 2015). Therefore, a better understanding of the source and properties of tissue-specific stem/progenitor cells could facilitate their therapeutic use.

Our attempts to isolate reproducibly homogenous population of cells from hUC led us to dissect the cord into at least three distinct sites, CPJ, CT, and WJ. Even though cells have been isolated from explants of CT, WJ and cord vein (Baksh et al., 2007; Chen et al., 2012; Gonzalez et al., 2010; Han et al., 2013; Hendijani et al., 2014; Kadivar et al., 2006; Conconi et al., 2011; Sarugaser et al., 2005; Romanov et al., 2003), to our knowledge this is the first report to show isolation of cells from CPJ. In our study, all hUC samples (n = 50) dissected into CPJ, CT and WJ yielded colony-forming cells with extensive proliferative capacity, depending on the site from which they were derived. Cells isolated from all three sites of hUC, had similar fibroblastoid morphology and expressed markers found on many tissue-specific stem/progenitor cells such as, CD29, CD44, CD73, CD90 and CD105. They were also positive for HLA-ABC but not HLA-DR. These results were in agreement with the previous reports (Dominici et al., 2006; Durnaoglu et al., 2011; Mennan et al., 2013; Sarugaser et al., 2005; Weiss et al., 2006). hUC cells were also found to express three markers (STRO-1, p75NTR, SUSD2) expressed by BMSCs (Lin et al., 2011; Sivasubramaniyan et al., 2013).

However, it is now understood that while tissue-specific stem/progenitors do express CD29, CD44, CD73, CD90 and CD105, not all cells that express these markers are stem/progenitor cells. Despite the expression of a number of SSC/BMSC-related genes, hUCs may be cord-specific stem/progenitor cells, however, unequivocal identification of hUC cells warrant further investigation. The hUC cells derived from CPJ, CT and WJ displayed multi-lineage properties when incubated in specific differentiation media in vitro. The differentiated derivatives of hUC cells displayed morphological and biochemical characteristics including expression of markers for chondrogenic (ACAN, SOX9 and COL2), osteogenic (COL1, OCN, OPN and RUNX2) and adipogenic (CEBP β , FABP4, and PPAR γ) lineages in vitro.

hUC cells expressed pluripotent markers, OCT4, NANOG, KLF4 and SOX2, but expression levels of all, except KLF4 were significantly lower than human ESCs (Fig. S3). Nevertheless, our results were in agreement with a previous report showing expression of pluripotency markers in cells from peri-natal sources (Nekanti et al., 2010). The results indicated that expression of these markers was site-dependent, as highest levels of OCT4 and NANOG were found in CPJ-hUC cells. These cells were also found to be the most proliferative when compared to CT- and WJ-hUC cells. However, despite their common characteristics, hUC cells derived from different sites of hUC differed in their colony forming efficiency, regulation of cell cycle, and proliferation capabilities.

Furthermore, comparative analysis of the isolated hUC cells showed that passaging gradually affected their properties. The cell size, CFE, and growth rate was reduced in case of CT- and WJ-hUC cells upon passaging compared to the CPJ-hUC cells. Passaging also affected the expression of pluripotent markers as well as certain cell surface markers. Comparison of LP and HP hUC cells showed not only reduction in expression of pluripotency and cell surface markers, but increased the cell size, suggesting that the cells are undergoing gradual changes

upon passaging. This hypothesis was strengthened by the fact that HP hUC cells had higher expression of CD105, which is known to be expressed in differentiating cells (Campbell and Pei, 2012; Habip Akbulut et al., 2012; Jiang et al., 2010).

Previous studies (Cheng et al., 2011; Zaim et al., 2012) have suggested that long-term passaging of stem/progenitor cells from different tissues causes senescence and the patterns of senescence differed between tissue sources and are age-dependent. Our analysis of HP hUC cells for negative Gal staining (see Fig. S4) and lack of upregulation of senescence markers (data not shown), such as p53 and p21 did not show that cells were undergoing senescence. Our findings are similar to an earlier report (Hass et al., 2011) which showed that the cells derived from neonatal tissues displayed no sign of cellular senescence over long-term culture. Nevertheless, additional studies on the type and cause of growth arrest in these cells should help develop long-term culturing techniques.

Further analysis of HP hUC cells showed that they expressed elevated levels of chondrogenic markers such as SOX9 and COL2, indicating that they were undergoing differentiation by expressing specific proteins found in chondrocytes. These findings were consistent with the microarray analysis showing the increased expression of COL12A1 in HP hUC cells. In fact, COL12A1 is reported to be associated with the COL2 and COL1 in the production of the extracellular matrix suggesting that HP hUC cells were undergoing chondrogenic differentiation (Dharmavaram et al., 1998; Gregory et al., 2001; Narcisi et al., 2015; Taylor et al., 2015).

In addition, the genome wide transcriptome analysis also revealed that there were various genes involved in cell proliferation and differentiation that were differentially expressed between the hUC cells derived from different sites of the same hUC. Out of these, two genes (ANXA3 and DD43) were expressed at significantly higher levels in CPJ- compared to CT- and WJ-hUC cells. These genes have been extensively studied as biomarkers in the prognosis of various kinds of cancer cell lines (Ambrosini et al., 2014; Zhai et al., 2014). The upregulation of these genes correlates with increased cell proliferation and self-renewal. In addition, two genes (HOXA6 and HOXD10) were expressed at lower levels in CPJ- compared with CT- and WJ-hUC cells. Both of these genes have been implicated in the regulation of cell differentiation (Osborne et al., 1998; Walters et al., 1997). Their lower expression could be responsible for longer-term proliferation as observed in case of CPJ-hUC cells. Interestingly, one gene (MAEL) was found to be expressed at high levels in CPJ-hUC cells. MAEL has been reported to have an essential role in spermatogenesis and transposon repression (Yuan et al., 2014). Investigation of the potential role of MAEL in hUC cells should be of interest.

Overall, ten genes, five of which are coding genes (FMOD, SULF1, COL12A1, STMN2 and MAEL), and five long-non-coding RNA (lncRNA) genes, were differentially expressed in hUC cells. lncRNAs are non-protein coding transcripts longer than 200 nucleotides. They have been implicated in the regulation of gene expression at epigenetic, transcriptional and post transcriptional levels (Cao, 2014). Recently, their role in many biological processes, including pluripotency and differentiation, has been recognized. Further investigation of the lncRNAs found in our study may shed light as to their specific functions in hUC cells.

While these results are based on in vitro studies, further in vivo studies are warranted for a more explicit proof for the ability of hUC cells exhibit multipotency and the ability to self-renew as would be demonstrated by serial transplantation assays.

In conclusion, our study provided a direct and relevant comparison of hUC cells isolated from the three different sites of hUC, namely the CPJ, CT, and WJ. The results clearly showed that CPJ is a distinct hUC

Fig. 7. Comparative microarray analysis between low and high passage CPJ-, CT-, and WJ-hUC cells. A: Venn diagram displaying the differentially expressed genes in LP vs. HP hUC cells. B-C and D-E: Venn diagrams and heatmaps showing differentially expressed categories of proliferation and differentiation genes, respectively. Up and downregulated genes are displayed in red and blue, respectively. Expanded heatmaps are presented in Figs. S9 and 10.

region with higher proliferation and differentiation potential, which could provide a promising source of stem/progenitor cells tissue engineering and regenerative medicine.

Disclosures

The authors indicate no potential conflicts of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.scr.2016.04.010>.

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