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ORIGINAL ARTICLE

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A comparative study of growth and osteogenic/adipogenic differentiation of human Wharton's Jelly stem cells under xenogenic and xeno-free culture conditions

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Keywords

Mesenchymal stem cells, Wharton's Jelly, xeno-free culture, valproic acid, multilineage differentiation

ABSTRACT • Background and Aims: Multipotent stem cells derived from human Wharton's Jelly stem cells have provided exciting prospects for stem cell-based treatment in many disorders. Since use of fetal bovine serum in cell culture may cause zoonotic infections and allergic side effects upon clinical use, the effect of human platelet rich plasma, as alternative of fetal bovine serum, on human Wharton's Jelly stem cell growth, tissue specific marker expression and differentiation was studied in a comparative manner. **Materials and Methods:** Human Wharton's Jelly stem cells were grown in media supplemented with either fetal bovine serum or human platelet rich plasma and their cell surface and intracellular markers compared by flow cytometry and immunocytochemistry, their growth characteristics compared by cell cycle and senescence analyses and their potential to differentiate into osteoblasts and adipocytes were evaluated. A histone deacetylase inhibitor, valproic acid, was used to augment cell differentiation. **Results:** Human Wharton's Jelly stem cells grown under both conditions expressed similar surface antigens, such as CD90, CD73, CD44, CD29 and HLA-ABC which specify their mesenchymal character, and did not express hematopoietic markers, like CD34 and CD45. Higher fraction of the human Wharton's Jelly stem cell population expressed CD11b, CD19 and CD71 under xeno-free conditions. Human Wharton's Jelly stem cells were found to express higher amounts of alpha-smooth muscle actin, fibronectin and nestin under xeno-free culture conditions. Human Wharton's Jelly stem cells precultured in xeno-free conditions deposited higher amount of CaP mineral upon osteogenic induction. Osteogenesis was augmented by valproic acid only in human Wharton's Jelly stem cells precultured under xenogenic conditions. Valproic acid repressed adipogenic differentiation. **Conclusion:** Human platelet rich plasma when used in place of fetal bovine serum in the human Wharton's Jelly stem cell culture, preserves their mesenchymal stem cell characteristics, positively contributes to cell growth kinetics, slows down cellular senescence, augments their neuro-regeneration-associated potency and also bone mineral deposition under osteogenic conditions.

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INTRODUCTION

Pluripotent stem cells are capable of differentiating into cells of many tissue types under suitable conditions, and thus show great treatment potential for tissue malfunctions or injuries (1,2). Embryonic stem cells (ESCs) derived from blastocyst stage embryos have the potential to differentiate into cells of all the three germ layers - endoderm, mesoderm and ectoderm - but there are ethical and practical issues (formation of teratoma when implanted to the body in an undifferentiated form, and trigger of immune response) related with their use in humans (3). Adult stem cells, either hematopoietic or mesenchymal, were previously thought to differentiate only to cells of the tissue that they were isolated from. However, increasing publications support their transdifferentiation into tissues different from their origin (2,4). They can be isolated not only from bone marrow but also from many other adult tissues.

Although bone marrow is considered a common source of autologous adult stem cells, its collection from the donor is not simple and practical as a routine method. Umbilical cord blood is a good source for stem cells; it is rich in hematopoietic stem cells (5) but mesenchymal stem cells (MSCs) are rare when compared to bone marrow (BM) (6,7). Other fetal tissues that link the baby to the mother, like placenta and umbilical cord matrix (Wharton's Jelly), are readily available tissues that house MSCs (8-10). Among these tissues, the source that is easiest to derive MSCs from is Wharton's Jelly (WJ), and the isolation method can either be through explant culture (11) or by enzymatic digestion (10,12). Previous studies with WJ MSCs indicate the possibility of their being more primitive relative to BM MSCs (10,12).

Mesenchymal stem cells (MSCs) from both the bone marrow and Wharton's Jelly can differentiate into osteoblasts, chondrocytes and adipocytes (12-15). They also have potential to differentiate into neurons, glial cells (16-24) and cardiomyocytes (12,25-29) and thus can be considered as a candidate cell source for the treatment of neural and cardiac malfunctions.

The rapid translation of mesenchymal stem cell research into clinical use has resulted in the development of cell-based strategies for multiple indications. However, limited supply of fetal bovine serum (FBS), its batch to batch compositional variation and its xenogeneic element capable of stimulating antibody mediated reactions and in some cases sensitization leading to anaphylaxis in the recipients create obstacles for its use in expansion of MSCs to therapeutic numbers (30,31). Therefore, an alternative xeno-free supplement with proper inherent growth promoting activities is demanded to yield homogenous cell populations with self-renewal and multi-lineage differentiation potential. Human autologous and allogenic supplements including platelet derivatives, platelet lysate (PL) and platelet-released factors and serum, are being assessed in MSC culture to replace FBS (32-35).

The effect of allogenic human platelet rich plasma [human platelet lysate (HPL)], a strong alternative of FBS, on human Wharton's Jelly stem cell (hWJ SC) growth, tissue specific marker expression and differentiation were evaluated in this study in a comparative manner. hWJ SCs belonging to the same donor were grown under the same culture conditions, but in presence of either FBS or HPL, to compare their cell surface markers by flow cytometry, tissue specific intracellular markers by immunocytochemistry, their growth characteristics by cell cycle and senescence analyses and their potential to differentiate into osteoblasts and adipocytes under inductive culture conditions. A histone deacetylase (HDAC) inhibitor, valproic acid, was tested for its potential to augment cell differentiation.

MATERIALS and METHODS

hWJ SC Isolation and Culture

Human Wharton's Jelly SCs (hWJ SCs) were isolated from umbilical cord matrix. Umbilical cords

were collected from full-term births with informed consent of the mothers, in accordance with the terms of the ethics committee of the University of Kocaeli, after either caesarean section or normal delivery and aseptically stored at 4°C in Hanks' balanced salt solution (HBSS) with 2% Pen/Strep until processing. The interval between collection and isolation of WJ SCs was at most 24 h. To isolate WJ SCs, the cords were rinsed several times with sterile saline, cut into 2-3 cm long segments and the vessels were stripped manually. The small explants (ca. 27 mm^3) were transferred to 6-well plates with a minimum amount of growth media to avoid floating. Tissue pieces from the same donor were transferred into separate 6-well plates and one of the following media formulations was used in the explant culture and afterwards: (1) αMEM with 10% fetal bovine serum, and 100 U/mL penicillin and 100 mg/mL streptomycin; (2) αMEM with 5% allogenic human platelet lysate (HPL), 2 mM L-glutamine, 2 U/mL heparin, and 100 U/mL penicillin and 100 mg/mL streptomycin.

The allogenic human platelet lysate (HPL) was prepared as follows: heparinized blood samples were layered over a Biocoll separating solution (density: 1.077 g/ml, Biochrom AG) for density centrifugation. The plasma layer and the buffy coat (low density mononuclear cells) were collected together in a tube and centrifuged at 1500 rpm for 5 min to precipitate the mononuclear cells. The supernatant composed of platelets and the plasma was collected and platelets lysed by two subsequent freeze-thaw cycles. The HPL was filter sterilized and stored as single use aliquots at -80°C. The explant cultures were left undisturbed for 2 weeks to allow migration of cells from the explants. WJ SCs were grown at 37°C in a humid atmosphere containing 5% CO_2 using the same media, subcultured upon reaching 70% confluence, and cryopreserved at passages 1 and 2 for future use.

Isolation and Culture of hBM-MSCs

Bone marrow aspirates (2-4 mL) were obtained from the iliac crest of patients (age= 2-7 years) with suspected idiopathic thrombocytopenic purpura (ITP). Informed consent was received in accordance with the terms of the ethics committee of the University of Kocaeli. Flow cytometric analyses confirmed that the donors were healthy. The bone marrow was diluted to 1:3 with phosphate buffered saline (PBS) and layered over a Histopaque-1077 (1.077 g/ml, Sigma-Aldrich, St. Louis, MO) for gradient centrifugation. The low density mononuclear cells were collected, washed twice with PBS, counted, and plated in tissue culture flasks at a density of 1.4 x 10^5 cells/cm^2 in MEM-Earle medium (Invitrogen/GIBCO, Grand Island, NY) containing 15% fetal bovine serum (FBS), 100 IU/ml penicillin, and 100 mg/ml streptomycin. The cells were incubated at 37°C in a humid atmosphere containing 5% CO_2 for 3 days. On the third day, red blood cells and other non-adherent cells were removed and fresh medium was added to allow further growth. The adherent cells were grown to 70% confluence and passaged at this stage not to impede their proliferative capacity.

Phenotype Identification

Flow cytometry: To confirm that hWJ SCs maintain their MSC phenotypic characteristics after growth in culture, undifferentiated cells grown in media containing either FBS or HPL were subjected to flow cytometric analysis. After the 3rd passage, the cells were harvested and resuspended in their own culture medium at a concentration of 1x10^6 cells/mL. Flow cytometry was performed using a FACSCalibur (Becton Dickinson, San Jose, CA).

The data were analyzed with Cell Quest software (BD Biosciences) and the forward and side scatter profiles were gated out of debris and dead cells.

Immunophenotyping of hWJ SCs was performed with antibodies against the following human antigens: CD3 (T-cell receptor; PerCP), CD4 (T-helper cell reseptor, FITC), CD5 (T1; Ly-1 signaling molecule, FITC), CD7 (thymocytes and mature T cell reseptor, FITC), CD8 (cytotoxic T-cell receptor;

PE), CD10 (N-cadherin/common leukocyte lymphocytic leukemia antigen-CALLA; PE), CD11b (Mac-1 a; integrin aM chain; PE), CD13 (aminopeptidase N/Vcadherin; PE), CD14 (monocyte differentiation antigen/LPS receptor; FITC), CD15 (3-fucosyl-N-acetyl-lactosamine; PE), CD19 (PerCP-Cy5.5), CD29 (Integrin b1 chain; PE), CD33 (sialic acid-binding immunoglobulin-like lectin 3; SIGLEC3; a surface marker for very early bone marrow derived hematopoietic stem cells; PE), CD34 (hematopoietic progenitor cell antigen; PE), CD44 (Hyaluronate/lymphocyte homing associated cell adhesion molecule-HCAM; PE), CD45 (Protein tyrosine phosphatase, receptor type, C/PTPRC/ leukocyte common antigen/cell marker of hematopoietic origin; FITC), CD71 (transferrin receptor; PE), CD73 (5'-nucleotidase, ecto; NT5E/integrin b5; PE), CD90 (Thy-1/ Thy-1.1-FITC), CD106 (vascular cell adhesion molecule (VCAM-1), FITC), CD117 (KIT or C-kit receptor/hematopoietic stem cells; PE), CD123 (Interleukin 3 resceptor a chain; PE), CD138 (Syndecan-1; PE), CD146 (melanoma cell adhesion molecule; MCAM/a marker for endothelial cell lineage and mesenchymal stem cells; PE), CD166 (activated leukocyte cell adhesion molecule; ALCAM/integrin a3; mesenchymal stem cell marker; PE), and HLA-DR (major histocompatibility complex, MHC class II, cell surface receptor; FITC), HLA-ABC (major histocompatibility class I antigen reseptor; PE), HLA-G (HLA Class I molecule, FITC). All of the antibodies were obtained from BD Biosciences, San Diego, CA. Cells from 3 donors were analysed for their cell surface markers.

Immunocytochemistry: To identify cellular markers, passage 4 cells of xenogenic and xeno-free cultures from the same donor were seeded on polyethylene coverslips (SARSTEDT), cultured for another 1–2 days in their own media and subjected to immunocytochemistry. Immunocytochemical analysis was performed using the streptavidin–peroxidase method (UltraVision Plus Large Volume Detection System Anti-Polyvalent, HRP immunostaining Kit; Thermo Scientific, Cheshire, UK). The cultured cells were fixed in ice-cold methanol for 10 min and allowed to dry. After additional PBS washes, cells were incubated with Ultra V Block for 5 min at room temperature. Then, cells were incubated overnight at 4°C with the primary antibodies specific to alpha-smooth muscle actin (α-SMA) (Thermo Scientific), desmin (Thermo Scientific), fibronectin (Santa Cruz Biotechnology), glial fibrillary acidic protein (GFAP) (Santa Cruz Biotechnology), nestin (Santa Cruz Biotechnology), and vimentin (Santa Cruz Biotechnology). The following day, cells were incubated with biotinylated secondary antibodies for 15 min at room temperature. Incubations were followed by streptavidin peroxidase treatment for 15 min at room temperature and signals were detected with the aminoethyhl carbazole (AEC) kit (Zymed Laboratories/Invitrogen, Carlsbad, CA). The cells were counter-stained with hematoxylin (Santa Cruz Biotechnology) and examined under light microscope (Leica DMI 4000B, Wetzlar, Germany).

Determination of Growth Characteristics

Cell cycle analysis: The cell cycle analysis was performed on the cells from xenogenic and xeno-free cultures by using the Cycle TEST PLUS DNA Reagent kit (Becton Dickinson, San Jose, CA) according to the manufacturer's instructions. To perform the assay $1x10^6$ cells were collected and resuspended in 1 mL of buffer solution. Data were acquired on a FACSCalibur flow cytometer using the Cell Quest software (BD Biosciences) and analyzed with a standard procedure using the CellFlT software. At least $2x10^4$ listmode data events were acquired for each sample.

Cellular senescence test: The amount of senescent cells was determined in the passage 3 hWJ SC cultures by using the Senescence Cells Histochemical Staining Kit (Sigma-Aldrich, St. Louis, MO) and propidium iodide (PI; Sigma) as fluorescent counterstain. The cells were seeded at a density of $1x10^4$ cells/cm^2 into 24-well plates and

cultured for 48 h before senescence associated β-galactosidase (SA-b-gal) staining. At the end of the staining procedure, four representative images were taken from diverse areas of each cell culture using phase-contrast microscopy and fluorescence microscopy. For the calculation of the percent senescent cells in the culture, the total number of cell nuclei and number of cell nuclei surrounded by cyan dye were enumerated.

In Vitro Differentiation

Adipogenic differentiation: Cells from passage 3 or 4 of hBM MSCs and hWJ SCs from xenogenic and xeno-free cultures were seeded at a density of $3x10^3$ cells/cm^2 onto fibronectin coated coverslips (BD Biosciences) in 6-well plates to induce adipogenic differentiation. The cells were cultured with the adipogenic medium, Minimum Essential Medium (MEM) (Invitrogen/GIBCO) supplemented with 10% FBS (Invitrogen/GIBCO), 0.5 mM isobutyl-methylxanthine (IBMX; Sigma–Aldrich), 10^{-6} M dexamethasone (Sigma–Aldrich, Fluka Chemie AG, Buchs, Switzerland), 10 mg/ml insulin (Invitrogen/GIBCO), 200 mM indomethacin (Sigma–Aldrich), and 1% antibiotics (Pen/Strep), for 3 weeks. 2 mM valproic acid was included in the adipogenic media of half of the samples. The medium was replaced twice a week. The presence of intracellular lipid droplets, which indicate adipogenic differentiation, was confirmed by Oil Red O (Sigma–Aldrich) staining, where 0.5% oil red O prepared in methanol was used.

Osteogenic differentiation: Cells from passages 3 or 4 of hBM MSCs and hWJ SCs from xenogenic and xeno-free cultures were seeded in 6-well plates with a type I collagen coated coverslip in each well at a density of $3x10^3$ cells/cm^2. For osteogenic differentiation, MEM (Invitrogen/GIBCO) was supplemented with 10 nM dexamethasone (Sigma–Aldrich), 50 µg/mL ascorbic acid 2-phosphate (Sigma), 10 mM β-glycerophosphate (Sigma–Aldrich), 1% antibiotics (Pen/Strep), and 10% FBS (Invitrogen/GIBCO) and the cells were incubated in this medium for up to 5 weeks. 2 mM valproic acid was included in the osteogenic media of half of the samples. The medium was replaced twice a week. Osteogenic differentiation was assessed via staining with Alizarin Red S (Sigma–Aldich, Fluka Chemie AG, Buchs, Switzerland).

For Alizarin Red staining, the cells were fixed for 5 min at room temperature in ice-cold 70% ethanol and then allowed to dry completely. The cells on the coverslips were stained with Alizarin Red solution composed of 2% Alizarin Red S (pH value of the Alizarin Red S solution was adjusted to 4.1–4.3 with ammonium hydroxide) for 30 s to 1 min, then washed with distilled water (20 dips). Excess dye was poured out and stained cells were dehydrated in acetone (20 dips), fixed in acetone–xylene (1:1) solution (20 dips), cleaned with xylene (20 dips), dried completely, and mounted in mounting medium.

RESULTS

Flow Cytometry- Cell Surface Marker Expression in hWJ SCs

Defined markers that specifically identify MSCs, and additional markers considered to be of importance were utilized to define the immunophenotypic characteristics of the cultured cells. Staining above 10% was considered as positive. Our data indicated that hWJ SCs expressed CD10, CD13, CD29, CD44, CD73, CD90, CD146, CD166, HLA-G and HLA-A,B,C but not CD3, CD4, CD5, CD7, CD8, CD14, CD15, CD33, CD34, CD45CD106, CD117, CD123, CD138 or HLA-DR under both culture conditions (Figure 1). These findings are consistent with the immunophenotypic mesenchymal stem cell characteristics mentioned in the literature (36) and provide some additional information. Culturing hWJ SCs in HPL supplemented media led to increase in population fraction expressing CD11b (macrophage-1 antigen; integrin alpha M chain), CD19 (B- lymphocyte lineage marker) and CD71 (transferrin receptor).

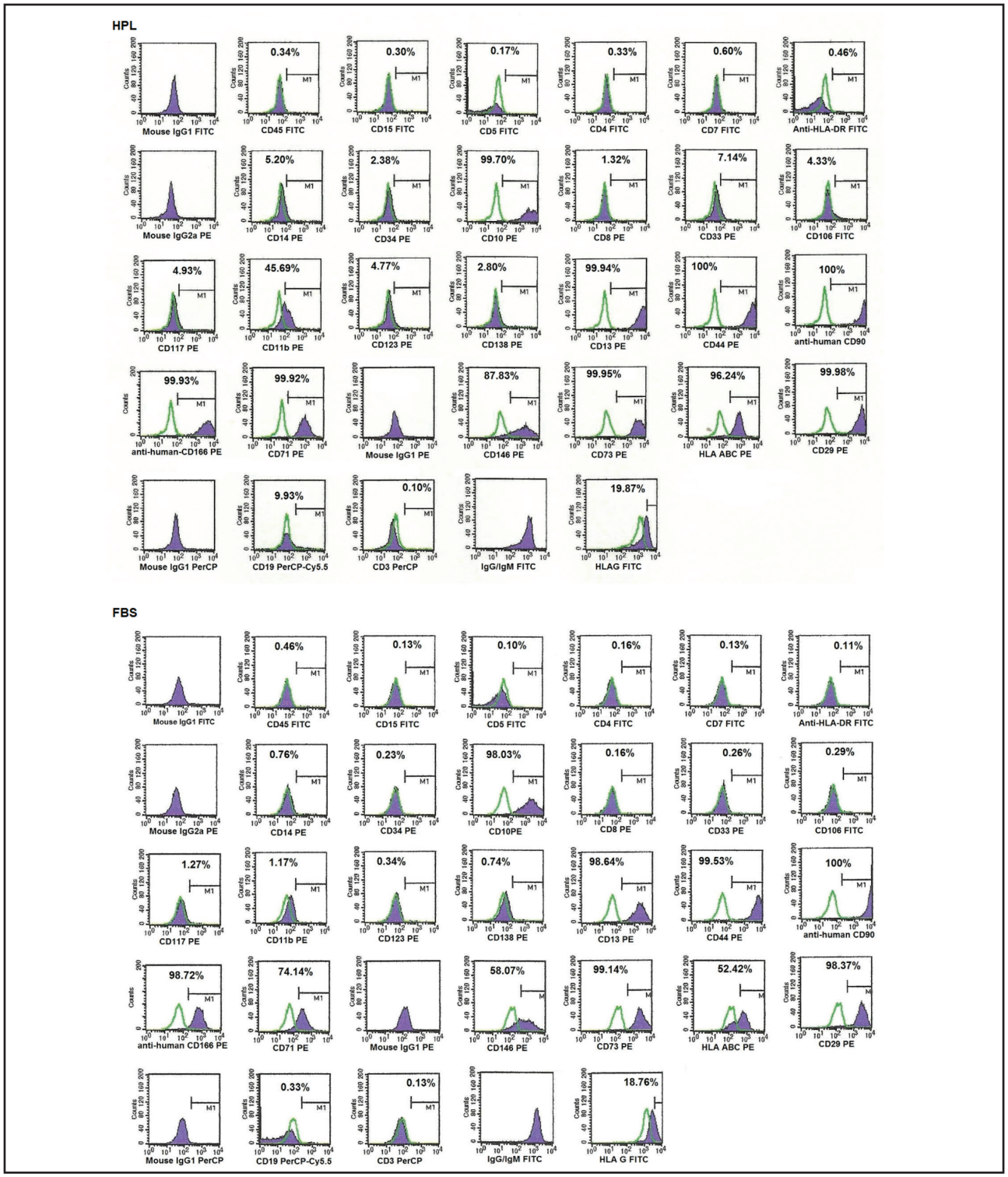

Figure 1 Representative flow cytometric histograms of cell-surface markers of hWJ SCs cultured either in presence of HPL or FBS at passage 3; cells were labeled with antibodies against various cluster of differentiation markers including hematopoietic and MSC markers or immunoglobulin isotype antibodies (Green line: histogram of isotype control immunoglobulin).

hWJ SCs: Human Wharton's Jelly stem cells. HPL: Human platelet lysate. FBS: Fetal bovine serum. MSC: Mesenchymal stem cell.

Immunocytochemical Properties of hWJ SCs

Figure 2 depicts immunocytochemical staining patterns of hWJ SCs cultured under xenogenic and xeno-free culture conditions. When cultured in media supplemented with FBS, the cells expressed GFAP (an intermediate filament protein specific for astroglial cells) and nestin, and also α-SMA and desmin to the lesser amount, but not fibronectin. Use of HPL in the growth media of the hWJ SCs from the same donor led to considerable increase in the expression of α-SMA, fibronectin and nestin, while downregulating desmin and GFAP expression. Vimentin, known as the intermediate filament specific to mesenchymal stem cells, was expressed in equal amounts under both culture conditions.

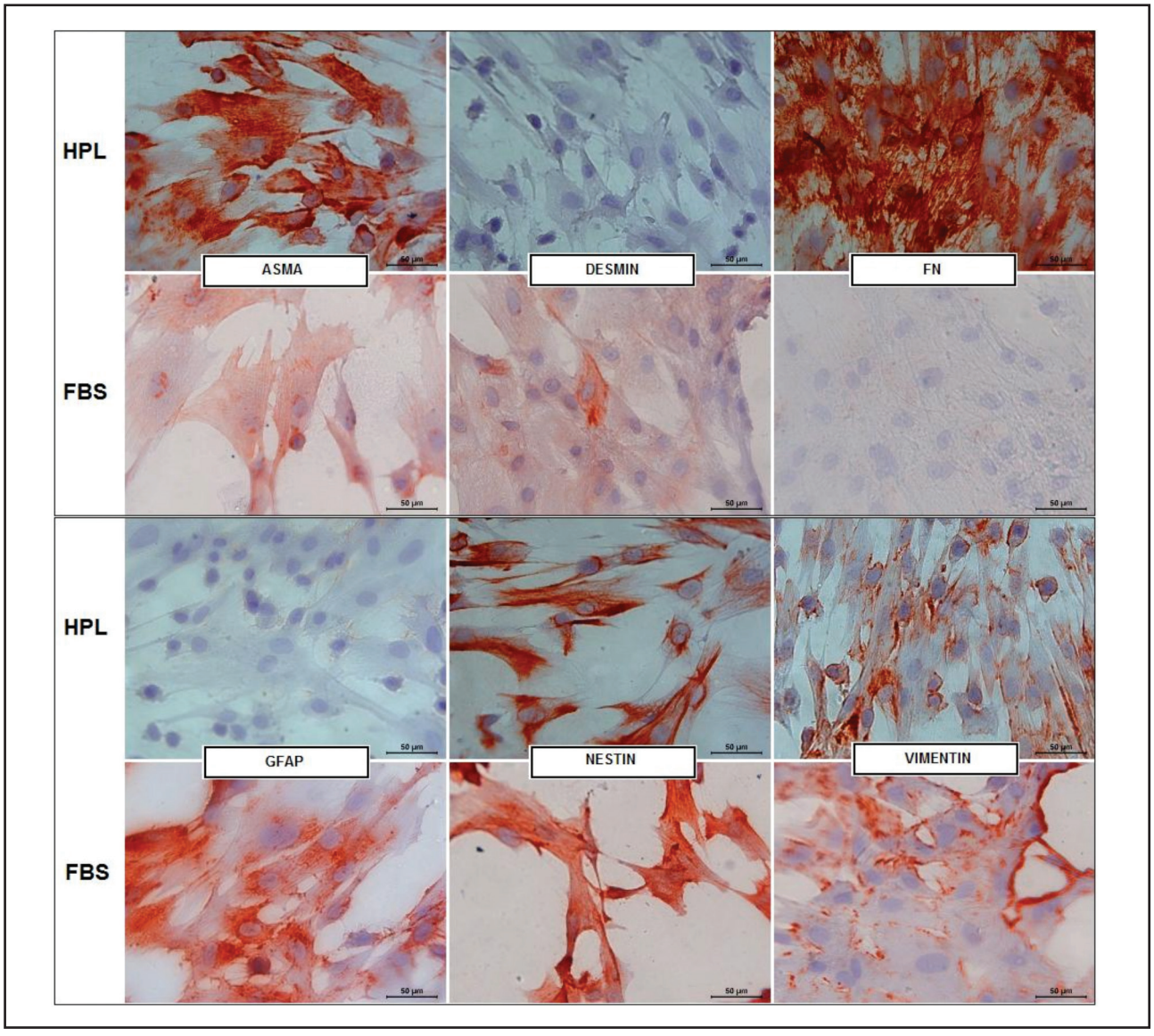

Figure 2 Immunophenotype of cultured hWJ SCs. Studies based on immunoperoxidase reactivity were performed on 3rd passage hWJ SCs cultured either in presence of HPL or FBS. Nuclei were counterstained with haematoxylin. Scale bar: 50 µm. ASMA : Alpha smooth muscle actin, FN: Fibronectin, GFAP: Glial fibrillary acidic protein.

hWJ SCs: Human Wharton's Jelly stem cells. HPL: Human platelet lysate. FBS: Fetal bovine serum.

Cell Cycle Analysis

Flow cytometric analysis of propidium iodide stained passage 4 hWJ SCs, when compared to mononuclear cells from peripheral blood of healthy humans (control), revealed their diploid DNA content (Figure 3). The DNA content of (G2/M)-phase subpopulation was twice the DNA content of G1-phase subpopulation. The distribution of G1 (2C DNA content), S (between 2C and 4C), and G2/M (4C DNA content) phase cells in the cell cycle was 92.35%, 7.08%, and 0.57%, respectively, when grown in media with FBS, and 91.01%, 6.76%, and 2.23%, respectively, when grown in media supplemented with HPL.

Cellular Senescence

Thin fibroblastic cells were present at the early passages of hWJ SCs under both culture conditions, where some cells acquired a more flattened morphology and became senescence associated β-galactosidase (SA-b-gal) positive (Figure 3). For the calculation of the percent senescent cells, the number of cell nuclei surrounded by the cyan dye (SA-b-gal positive cells) was divided by the total number of cell nuclei and multiplied by 100. 3.29 % of hWJ SCs grown in presence of HPL showed a senescent phenotype, while 4.45 % of the cells appeared to be senescent when grown in FBS supplemented media.

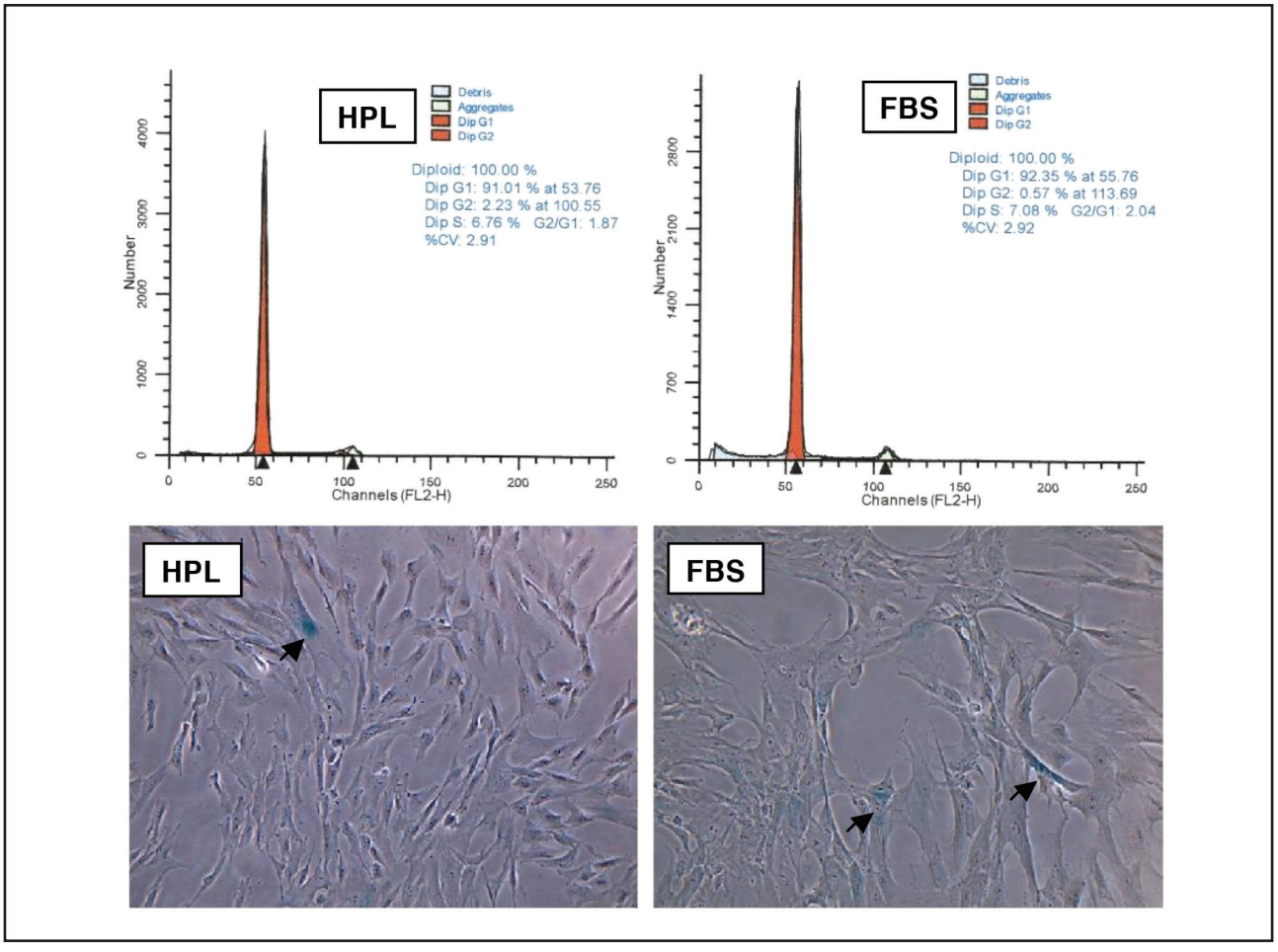

Figure 3 Cell cycle analysis and cellular senescence assay results of hWJ SCs grown under zenogenic (FBS) or zenogenic-free (HPL) culture conditions. β-Gal positive senescent cells are pointed with black arrows.

hWJ SCs: Human Wharton's Jelly stem cells. HPL: Human platelet lysate. FBS: Fetal bovine serum.

Differentiation potential of hWJ SCs

Histochemical methods were used to determine the potential of hWJ SCs to differentiate into adipogenic and osteogenic lineages. hBM MSCs cultured in FBS containing media were used as positive controls in these differentiation experiments.

Fluorescence micrographs of the neutral lipid vacuoles in the adipocytes after Oil Red O staining revealed that hWJ SCs are able to synthesize smaller lipid droplets when compared to hBM MSCs. Valproic acid repressed adipogenic differentiation in both cell types, but even more in hWJ SCs precultured under xeno-free conditions.

Alizarin Red S staining of the hBM MSCs and hWJ SCs differentiated into osteogenic lineage revealed the calcified nodules (Figure 4). While it took only 2 weeks to obtain the calcified bone nodules in the

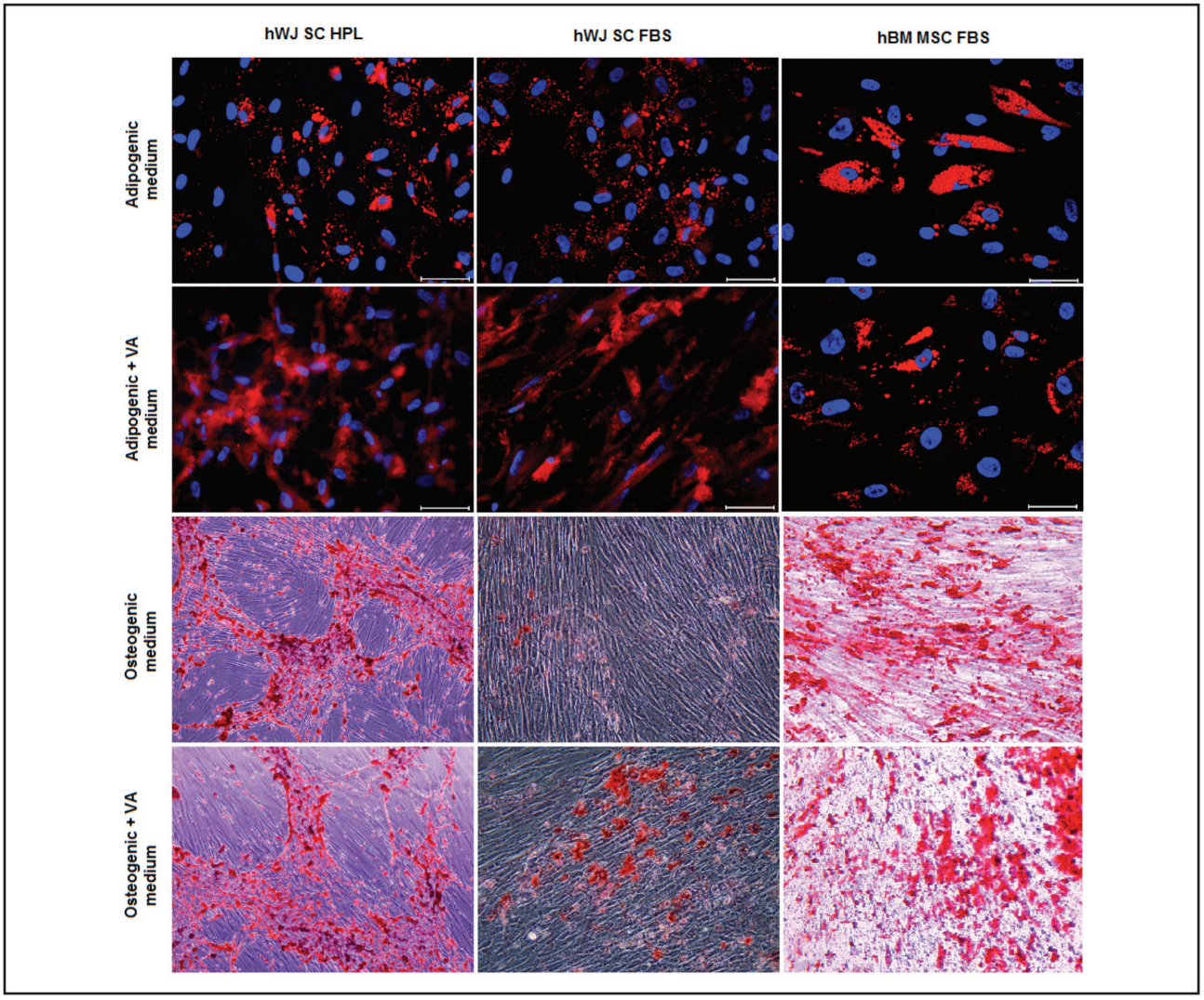

Figure 4 Microscopic images of hWJSCs and hBM-MSCs differentiated to adipocytes and osteoblasts. The neutral lipid vacuoles in the adipocytes were revealed by Oil Red O staining and subsequent fluorescence microscopy (red: lipid vacuoles; blue: nuclei). Phase-contrast micrographs of the hBM-MSCs and hWJ SCs differentiated into osteogenic lineage show the calcified nodules stained with Alizarin Red S (magnification: hWJ SC HPL: x100, hWJ SC FBS & hBM MSC FBS: x200).

hWJ SCs: Human Wharton's Jelly stem cells. hBMMSc: Human bone marrow mesenchymal stem cells. HPL: Human platelet lysate. FBS: Fetal bovine serum. VA: Valproic acid.

osteogenic culture of hBM MSCs, this time period was longer, usually between 3 to 5 weeks, in case of hWJ SCs. hWJ SCs precultured under xeno-free conditions deposited higher amount of CaP mineral when compared to their counterparts grown in FBS supplemented medium.

Use of valproic acid during the osteogenic culture of hWJ SCs precultured in FBS containing media significantly improved the bone mineral deposition. On the other hand, improvement of osteogenesis by valproic acid was not observed in the osteogenic culture of hWJ SCs precultured under xeno-free conditions.

DISCUSSION

The use of fetal bovine serum during in vitro expansion of MSCs might pose a potential hazard to recipients in a clinical use due to xenogenic proteins internalized in stem cells. Transmission of viral/prion disease or induction of immunological reactions by these xenogenic proteins is the major obstacle in such treatment strategies. To avoid such problems, replacement of FBS with human serum or platelet lysate can be considered as better choice for clinical applications.

The platelets and the plasma of human peripheral blood were used in our study to derive a platelet lysate by the freze/thaw cycles. The platelets are known to harbour mitogenic growth factors and molecules, like platelet-derived growth factor (PDGF), basic fibroblast growth factor (bFGF) and transforming growth factor beta (TGF-β), that promote tissue repair and angiogenesis (37). Explant culture of Wharton's Jelly was performed in a growth media containing either FBS or HPL to isolate the hWJ SCs, which were characterized at passage numbers 3 or 4.

Human Wharton's Jelly stem cells (hWJ SCs) grown under xenogenic or xeno-free conditions expressed similar surface antigens that are typical for MSCs such as CD90, CD73, CD44, CD29 and HLA-ABC, and did not express hematopoietic markers, like CD34 and CD45. Higher fraction of the hWJ SC population expressed CD11b, CD19 and CD71 under xeno-free conditions, i.e. in HPL containing growth media. CD71 is defined as the transferrin receptor protein 1 (TfR1). Increase in the fraction expressing CD11b (macrophage 1 antigen) and CD19 (protein found on B cells and dendritic cells) suggest an increase in adherent white blood cell population or an increase in amount of cells expressing these markers.

Cell cycle analysis of the hWJ SCs revealed a hundred percent diploid population under both culture conditions. While percentage of cells at S (between 2C and 4C) phase was very similar under both conditions, the proliferation index (S+G2/M) of cells grown in HPL supplemented media was higher when compared to the cells grown in FBS supplemented media (8.99% vs. 7.65%). Cellular senescence was observed in a lower fraction of hWJ SCs cultured in HPL supplemented media. Both results, i.e. increase in proliferation index and decrease in senescent cell fraction, indicate an improvement in growth kinetics of hWJ SCs when grown in the xeno-free media.

Human Wharton's Jelly stem cells (hWJ SCs) were found to express higher amounts of alpha- smooth muscle actin (α-SMA), fibronectin and nestin, while being negative for glial fibrillary acidic protein (GFAP) and desmin under xeno-free culture conditions. α-SMA, initially thought to be specific to vascular smooth muscle cells, was verified too as a marker for human neural-crest stem cell lines in vitro (38). Increase in expression of both α-SMA and nestin, a specific neuro-ectodermal marker, strongly support the neuro-regeneration–associated potency of the hWJ SCs (39), a property enhanced when cultured in HPL supplemented media. To test the therapeutic value of undifferentiated hWJ SCs, Weiss et al. (2006) transplanted the cells into the brains of hemiparkinsonian rats that were not immune-suppressed and this treat-

ment ameliorated apomorphine-induced rotations in the pilot test (10).

Human Wharton's Jelly stem cells (hWJ SCs) precultured in either FBS or HPL supplemented media were further incubated in adipogenic and osteogenic media to compare their potential to differentiate into osteoblasts and adipocytes. There was no difference between adipogenic differentiation of hWJ SCs precultured in FBS or HPL supplemented media, but osteogenesis was considerably enhanced in hWJ SCs precultured in HPL supplemented media. Similar results were found in a study by Riordan et al. (2015) who cultured hWJ SCs in a media supplemented with XcytePLUS™, a human platelet lysate based product (40). A histone deacetylase (HDAC) inhibitor, valproic acid, was also used in the adipogenic and osteogenic media to augment cell differentiation. It has been previously demonstrated that valproic acid stimulates osteogenic differentiation of adipose tissue and cord blood derived stromal cells (41,42). Valproic acid is tested for the first time in our study with hWJ SCs. Cho et al. (2005) and Lee et al. (2009) reported osteogenesis results similar to our results with hWJ SCs precultured in FBS containing media; osteogenesis was enhansed in the MSCs when cultured with valproic acid (41,42). Lee et al. (2009) also reported that valproic acid decreased the efficiency of adipogenic differentiation, as it was the outcome in our study, too (42). When preculture was carried out in HPL supplemented media, subsequent differentiation experiments with valproic acid resulted in potentiation of osteogenesis and almost complete elimination of adipogenesis in hWJ SCs.

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