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Human Umbilical Cord Wharton's Jelly Mesenchymal Stem Cells Do Not Transform to Tumor-Associated Fibroblasts in the Presence of Breast and Ovarian Cancer Cells Unlike Bone Marrow Mesenchymal Stem Cells

Arjunan Subramanian,[1] Gan Shu-Uin,[2] Ngo Kae-Siang,[2] Kalamegam Gauthaman,[1] Arijit Biswas,[1] Mahesh Choolani,[1] Ariff Bongso,[1*] and Fong Chui-Yee[1]

[1]*Department of Obstetrics and Gynaecology, Yong Loo Lin School of Medicine, National University of Singapore, Kent Ridge 119074, Singapore*

[2]*Department of Surgery, Yong Loo Lin School of Medicine, National University of Singapore, Kent Ridge 119074, Singapore*

ABSTRACT

Human bone marrow mesenchymal stem cells (hBMMSCs) were shown to transform into tumor-associated fibroblasts (TAFs) when in the vicinity of breast cancer tumors and played an important role in tumor enhancement and metastasis. In early human development MSCs migrating from the yolk sac and aorta-gonad-mesonephros (AGM) via the umbilical cord to the placenta and back to the fetal bone marrow were shown to get trapped in the gelatinous Wharton's jelly of the umbilical cord. The common origin of the Wharton's jelly MSCs and the finally homed hBMMSCs prompted us to evaluate whether hWJSCs are also involved in TAF transformation. hWJSCs and hBMMSCs were grown in the presence of breast and ovarian cancer cell conditioned medium (MDA-TCM, TOV-TCM) for 30 days. No changes were observed in the hWJSCs but the hBMMSCs transformed from short to thin long fibroblasts, their proliferation rates increased and CD marker expression decreased. The transformed hBMMSCs showed positive staining for the tumor-associated markers FSP, VEGF, EGF, and Tn-C. Real-time PCR and multiplex luminex bead analysis showed upregulation of TAF-related genes (FSP, FAP, Tn-C, Tsp-1, EGF, bFGF, IL-6, α-SMA, VEGF, and TGF-β) for hBMMSCs with low expression for hWJSCs. The luciferase assay showed that hWJSCs previously exposed to MDA-TCM or TOV-TCM had no stimulatory growth effect on luciferase-tagged MDA or TOV cells unlike hBMMSCs. The results confirmed that hWJSCs do not transform to the TAF phenotype and may therefore not be associated with enhanced growth of solid tumors making them a safe MSC for cell based therapies. J. Cell. Biochem. 113: 1886–1895, 2012.

KEY WORDS: CANCER CELLS; HUMAN WHARTON'S JELLY STEM CELLS; TUMOR-ASSOCIATED FIBROBLASTS

It has been shown that the immediate neighboring vicinity of the tumor microenvironment is laden with a diverse variety of vascular and stromal cell types that participate in the molecular events leading to tumor invasion and metastasis [Wels et al., 2008]. The same workers reported that certain secretions of the tumor cells can stimulate the migration of host cells both at short and long distances from the primary tumor area and also encourage the movement of cells to distant tissues. This takes place throughout tumor growth and metastasis providing the basis for the systemic nature of malignancies [Wels et al., 2008]. There are various cell types in the tumor microenvironment such as fibroblasts, endothelial cells, blood cells and pericytes, and these cells interact with the tumor cells via the secretion of cytokines, hormones, chemokines, and proteases [Spaeth et al., 2009]. The fibroblasts that interact with the tumor cells were aptly referred to as tumor-associated fibroblasts (TAF) and from a functional point of view described as migratory neighbors and distant invaders [Wels et al., 2008]. There is ample evidence to demonstrate that TAFs are an important player in tumor formation, growth, enhancement and metastasis particularly for solid tumors [Cardone et al., 1997; Bissell

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*Correspondence to: Ariff Bongso, Professor, Department of Obstetrics and Gynaecology, Yong Loo Lin School of Medicine, National University of Singapore, Kent Ridge 119074, Singapore. E-mail: obgbongs@nus.edu.sg
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and Radisky, 2001; Blankenstein, 2005]. Recent studies show that breast cancer TAFs encourage tumor cell growth compared to fibroblasts obtained from benign non-tumorigenic microenvironments [Mishra et al., 2008].

One of the origins suggested for TAFs was migrating bone marrow-derived cells from the peripheral circulation to the tumor site [Jodele et al., 2005]. Other workers later reported that these bone marrow-derived cells were actually mesenchymal stem cells (MSCs) that had the potential to differentiate into TAFs when present within the tumor microenvironment [Spaeth et al., 2009]. These workers provided ample evidence to show that human bone marrow MSCs (hBMMSCs) acquired a TAF phenotype following exposure to or after systemic recruitment into adenocarcinoma xenograft models of breast, pancreatic, and ovarian cancers. They characterized the MSC-derived TAFs and reported via immunohistochemistry that they were positive for fibroblast specific proteins (FSP), fibroblast activated proteins (FCP), cell aggressive markers (tenascin-c, thrombospondin-1, stromelysin-1), tumor growth factors (HGF, EGF, IL-6), and angiogenic factors (alpha smooth muscle actin, desmin, VEGF). They further confirmed that hBMMSCs stimulated tumor growth primarily via the secretion of IL-6.

MSCs with properties that are different from those in the bone marrow have been isolated from birth-related tissues such as amnion [Illancheran et al., 2007], subamnion [Kita et al., 2010], umbilical cord blood [Musina et al., 2007], perivascular regions surrounding the umbilical blood vessels [Sarugaser et al., 2005] and from the Wharton's jelly [Fong et al., 2007, 2010a; Troyer and Weiss, 2008] within the umbilical cord. We have derived, characterized and studied in detail the nature and properties of human Wharton's jelly stem cells (hWJSCs) [Fong et al., 2007, 2010a]. These MSCs have a CD signature slightly different from human bone marrow MSCs (hBMMSCs), retain their stemness properties for longer periods of time in vitro compared to hBMMSCs (approximately 10 passages vs. 3 passages), can be differentiated into a variety of desirable tissues and are hypoimmunogenic [Fong et al., 2007, 2010a, 2011; Karahuseyinglu et al., 2007; Weiss et al., 2008; La Rocca et al., 2009]. hWJSCs do not induce teratomas in immunodeficient SCID mice and when transplanted into diseased animal models they engraft successfully and do not produce tumors [Fan et al., 2011; Gauthaman et al., 2011]. More interestingly, it was reported that rat WJSCs completely abolished rat mammary carcinomas after intra-tumoral or intravenous injection with no evidence of metastasis or recurrence after 100 days [Ganta et al., 2009]. Later, Ayuzawa et al. [2009] showed that unengineered hWJSCs significantly attenuated the growth of human breast cancer cells in vitro and intravenous hWJSC injections abolished human breast cancer tumor growth in the SCID mouse model. More recently, our group observed that hWJSCs inhibited the growth of not only breast cancer cells but also ovarian and osteosarcoma cells in vitro in varying degrees (Gauthaman et al., unpublished work). Such anticancer properties that appear to be unique to hWJSCs may be related to the upregulation of various tumor suppressor and pro-apoptotic genes observed in the transcriptomes of hWJSCs when their microarray transciptome analysis was compared with bone marrow MSCs, ESCs, and somatic cells [Fong et al., 2010b].

The common origin of hWJSCs and hBMMSCs and these unusual characteristics of hWJSCs prompted us to explore their transformation capabilities to TAFs with the hope that such information will throw light on the pathogenesis of solid tumors and also provide insights into the non-tumorigenic and anti-tumorigenic properties and safety of hWJSCs for clinical application.

MATERIALS AND METHODS

hWJSCs AND hBMMSCs

Human Wharton's jelly stem cells were obtained from discarded umbilical cords using our derivation methods [Fong et al., 2007, 2010a] after receiving informed patient consent and ethical approval from the Ministry of Health Institutional Domain Specific Review Board (DSRB), Singapore. Primary cultures of hWJSCs and passages were grown in hWJSC medium comprised of 80% DMEM high glucose, 20% fetal bovine serum (FBS) (Invitrogen Life Technologies, Carlsbad, CA), 1% non-essential amino acids (NEAA), 2 mM L-glutamine, 0.1 mM β-mercaptoethanol, antibiotic/antimycotic mixture, 1% insulin-transferrin-selenium (ITS), and 16 ng/ml basic fibroblast growth factor (bFGF) (Millipore Bioscience Research Agents, Temecula, CA). Commercial human bone marrow mesenchymal stem cells (hBMMSCs) were purchased from Lonza (Allendale, NJ) and approval for their use in this project was obtained from the National University of Singapore Institutional Review Board (NUS-IRB). Primary cultures of hBMMSCs were expanded in the commercial medium supplied with the cells by the manufacturer and frozen for subsequent experiments.

Early frozen passages of the hWJSCs and hBMMSCs (P2-P4) were thawed and grown in a simple basal medium of 90% DMEM high glucose, 10% heat inactivated FBS (Invitrogen Life Technologies), 1% non-essential amino acids (NEAA), 2 mM L-glutamine, and antibiotic/antimycotic mixture (Millipore Bioscience Research Agents) for all the experiments in this study.

CD MARKER ANALYSIS OF hWJSCs AND hBMMSCs

The hWJSCs and hBMMSCs were characterized using a range of CD markers. Briefly, the hWJSC and hBMMSC monolayers were dissociated with trypsin (TrypLE™ Express, Invitrogen Life Technologies) for 2–3 min, the cells washed with PBS (−) and then blocked with 10% normal goat serum (NGS) to prevent non-specific binding. The cells were incubated with primary antibodies (1:100) for several CD markers, viz. CD13, CD14, CD29, CD44, and CD105 (Biolegend, San Diego, CA) for 30 min. The cells were then washed to remove the primary antibodies and incubated with secondary antibody Alexa Fluor®488 (1:750) (Invitrogen Life Technologies) for 30 min. The cells were washed once again with PBS (−), filtered using a 60 μm nylon strainer (BD, Franklin Lakes, NJ) to remove the cell clumps and analyzed using a CyAn™ ADP Analyzer (Beckman Coulter, Fullerton, CA).

CANCER AND FIBROBLAST CELL LINES

Commercial cancer [human ovarian cancer (TOV-112D) and breast cancer (MDA-MB-231)] and genetically normal fibroblast [foreskin fibroblasts (CCD-1112sk)] cell lines were purchased from ATCC

(Manassas, USA) and approval for their use obtained from NUS-IRB. These cell lines were separately cultured in the same simple basal medium that was used above to grow the passaged hWJSCs and hBMMSCs for three passages before being used for preparation of tumor-conditioned medium (TCM) and fibroblast-conditioned medium (FCM).

PREPARATION OF TUMOR-CONDITIONED AND FIBROBLAST-CONDITIONED MEDIA (TCM, FCM)

The MDA-231, TOV-112D, and CCD-1112sk cell lines were separately cultured in T-75 flasks in simple basal medium. When the cells were 70–80% confluent, the old medium was removed from each flask and 10 ml of fresh simple basal medium added. After 16 h of growth of the cells in the simple basal medium, the medium was separated from the cells and called MDA-tumor conditioned medium (MDA-TCM), TOV-tumor conditioned medium (TOV-TCM), and CCD-fibroblast conditioned medium (CCD-FCM). All three conditioned media were filtered using a 0.22 μm filter (Millipore Bioscience Research Agents) before being used or stored at 4°C. A 100% concentration (undiluted) of each of the conditioned media was used for the culture of hWJSC or hBMMSCs.

EXPOSURE OF hWJSCs AND hBMMSCs TO TCM AND FCM

hWJSCs and hBMMSCs (1×10^4 cells of each) (4P) were seeded into T75 flasks and grown in the presence of MDA-TCM and TOV-TCM (treatments), CCD-FCM (controls) and non-conditioned basal medium (BM) (controls) at 37°C in a 5% CO_2 in air atmosphere for 30 days with changes of culture media every 48 h. When confluent before day 30, the monolayers were trypsinized and re-seeded at 1×10^4 cells into T75 flasks for further propagation.

CELL PROLIFERATION (MTT ASSAY)

The cell proliferation rates of the hWJSCs and hBMMSCs grown in MDA-TCM, TOV-TCM and controls were analyzed on days 1, 10, 20, and 30. The cell proliferation assay was performed using a MTT reagent kit [3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide] (Sigma, St. Louis, MO) and according to the manufacturer's instructions. Briefly, 10 μl MTT reagent (0.5 mg/ml) was added to the culture medium and the cells incubated for 3–4 h at 37°C in a 5% CO_2 in air atmosphere. The medium was then removed and 100 μl of the detergent reagent supplied with the kit was added to the cells and incubation carried out in the dark for another 2 h. Absorbance at 570 nm was measured using a microplate ELISA spectrophotometer (μQuant, BioTek, Winooski, VT) with a reference wavelength of 650 nm.

QUANTITATIVE REAL TIME POLYMERASE CHAIN REACTION (qRT-PCR)

After 30 days of growth of the hWJSCs and hBMSCs in MDA-TCM, TOV-TCM and controls, total RNA was extracted using TRIzol™ reagent (Invitrogen Life Technologies). cDNA was prepared with random hexamers using the SuperScript™ first strand synthesis system (Invitrogen Life Technologies). GAPDH was used as the internal control. Primer sequences were taken from earlier published studies and are summarized in Table I. qRT-PCR analysis was performed using the ABI PRISM 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA) with SYBR green as previously described [Gauthaman et al., 2010] and relative quantification was performed using the comparative CT ($2^{-\Delta\Delta CT}$) method.

TABLE I. Primer Sequences of Genes

Gene name	Primer sequence
Tn-C	F: 5′-CAAGTTCAGCGTGGGAGATG-3′ R: 5′-ACTGGATTGAGTGTTCGTGGC-3′
FAP	F: 5′-TCAACTGTGATGGCAAGAGCA-3′ R: 5′-TAGGAAGTGGGTCATGTGGGT-3′
α-SMA	F: 5′-AGGGGGTGATGGTGGGAATG-3′ R: 5′-GCCCATCAGGCAACTCGTAAC-3′
TSP-1	F: 5′-CAGCAGCCGCTTTTATGT-3′ R: 5′-CCGAGTATCCCTGAGCCCTC-3′
HGF	F: 5′-CTGGTTCCCCTTCAATAGCA-3′ R: 5′-CTCCAGGGCTGACATTTGAT-3′
IL6	F: 5′-GAAGATTCCAAAGATGTAGCCG-3′ R: 5′-TGTTTTCTGCCAGTGCCTC-3′
FGF2	F: 5′-AGAGCGACCCTCACATCAAG-3′ R: 5′-ACTGCCCAGTTCGTTTCAGT-3′
VEGFA	F: 5′-CCCACTGAGGAGTCCAACAT-3′ R: 5′-AAATGCTTTCTCCGCTCTGA-3′
SDF1	F: 5′-AGAGCCAACGTCAAGCATCT-3′ R: 5′-CTTTAGCTTCGGGTCAATGC-3′
FSP1	F: 5′-GATGAGCAACTTGGACAGCA-3′ R: 5′-CTTCCTGGGCTGCTTATCTG-3′

IMMUNOCYTOCHEMISTRY

After 30 days of growth of the hWJSCs and hBMMSCs in MDA-TCM, TOV-TCM and controls the cells were fixed with 100% cold ethanol for 5 min, washed with PBS and blocked with 10% NGS for 15–20 min at room temperature. The cells were then incubated with mouse monoclonal primary antibodies (Abcam, Cambridge, MA) for VEGF (5 μg/ml), EGF (1 μg/ml), FSP (5 μg/ml), and thrombospondin (5 μg/ml) for 1 h. The cells were incubated with goat anti-mouse fluorescent secondary antibody (Alexa Fluor 488; 2 μg/ml) for 30 min, stained with 4′-6-diamidino-2-phenylindole (DAPI; 0.5 μg/ml) (Molecular Probes, Invitrogen Life Technologies) for 5 min at room temperature and then analyzed using fluorescence microscopy.

GROWTH FACTOR ANALYSIS VIA MULTIPLEX LUMINEX® BEADS ASSAY

After 30 days of growth of the hWJSCs and hBMMSCs in MDA-TCM, TOV-TCM and controls, the differential cytokine levels in 100 μl of MDA-TCM, TOV-TCM and controls were analyzed using the Bio-Rad Express assay kit for human VEGF, IL-6 and TGF-β (Bio-Rad Laboratories, Singapore Pte Ltd). The 96-well microtiter plate provided with the kit was washed with 100 μl of wash buffer and 50 μl of beads were added to each well. The samples were diluted in equal volumes of assay diluent and 50 μl of diluted sample added to each well. The assays were run in duplicate together with the standards and the plates incubated for 1 h at room temperature on a shaker in the dark. After incubation the wells were washed twice in buffer and 100 μl of secondary biotinylated antibody (1:10 dilution in biotin diluent provided with the kit) was added to each well. The plates were then incubated at room temperature for 1 h in the dark after which the wells were washed twice in buffer. Then 100 μl of Streptavindin-PE (provided with the kit) was added to each well and plates incubated for 30 min at room temperature in the dark. Finally,

the wells were washed thrice, each then filled with 100 μl of wash buffer and the plates incubated for 2–3 min at room temperature in the dark. The contents of each well were then passed through a Bio-plex array reader and data subsequently analyzed using the Bio-plex manager software, version 3.

LUCIFERASE ASSAY

To compare the proliferative effect of hWJSCs and hBMMSCs (with and without previous exposure to MDA-TCM or TOV-TCM) on MDA-MB-231 and TOV-112D cell numbers, the luciferase assay was carried out using the Steady-Glo (R) Luciferase assay kit (Promega) according to the manufacturer's instructions. Briefly, the TOV-112D and MDA-MB-231 cells were transduced with lentiviral vector encoding the firefly luciferase gene. hWJSCs and hBMMSCs (1×10^4 cells of each) with and without previous exposure to MDA-TCM or TOV-TCM for 30 days were cocultured with an equal number of luciferase-tagged TOV-112D or MDA-MB-231 cells in the wells of a black-walled, clear-bottom 24-well plate. On days 1, 3, and 5, 100 μl of Steady-Glo™ Reagent supplied with the kit was added to an equal volume cell suspension (100 μl) that was well-mixed with the culture medium in each well, and incubated for 10 min at room temperature to allow cell lysis. Luminescence was then measured using a VICTOR3™ Multilabel Counter (PerkinElmer).

STATISTICS

One-way ANOVA with Bonferroni's multiple comparisons post hoc analysis or Student's *t*-test with statistical package for Social Sciences (SPSS 13) was used to evaluate statistically significant differences between the cancer and control cells for the various

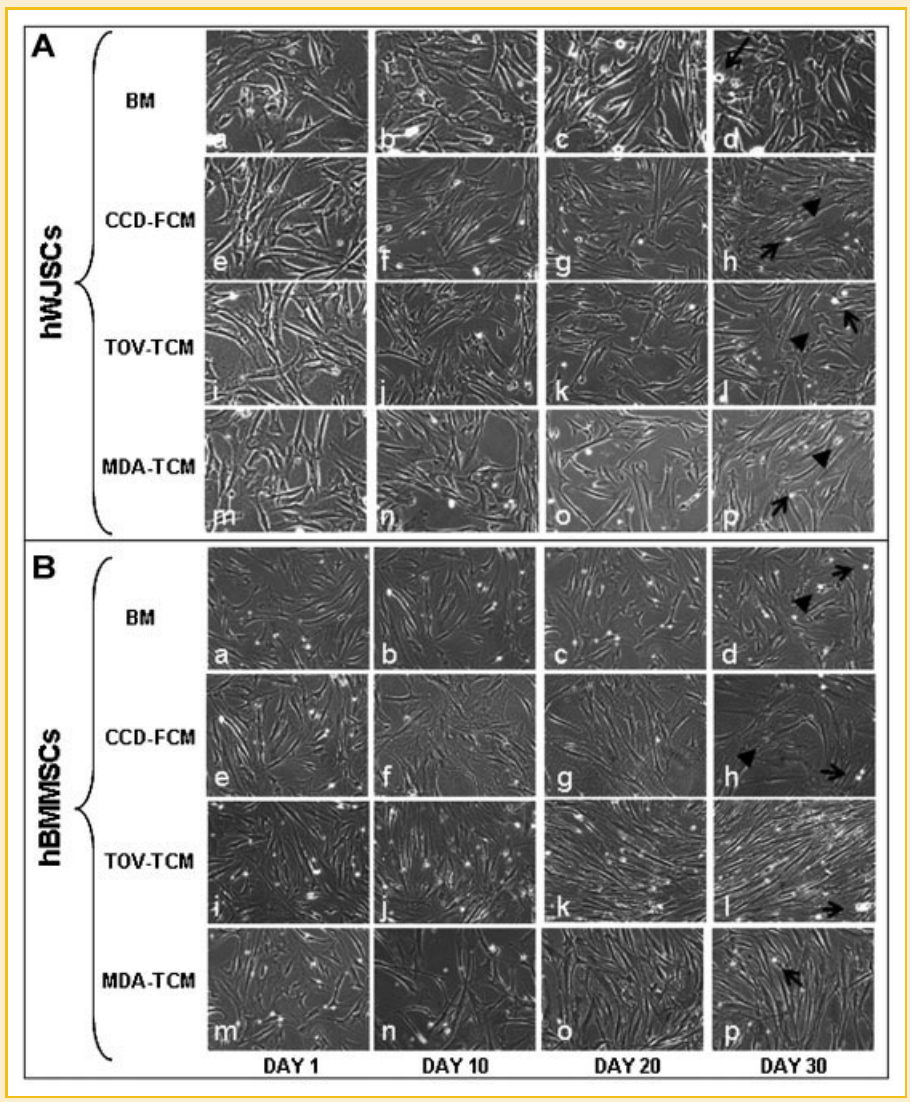

Fig. 1. Morphology of hWJSCs and hBMMSCs in the presence of TOV-TCM, MDA-TCM and controls. A (a–p): hWJSCs retained their morphology of short flat fibroblast-like cells in TOV-TCM, MDA-TCM and controls (BM, CCD-FCM). B: hBMMSCs retained their short fibroblast-like phenotype in controls (BM, CCD-FCM) (a–h) but changed to elongated spindle-shaped fibroblasts in TOV-TCM and MDA-TCM (i–p).

assays. Three different replicates were carried out for individual assays and the results expressed as mean ± SEM and a value of $P < 0.05$ was considered to be statistically significant.

RESULTS

MORPHOLOGY OF hWJSCs AND hBMSCs

The hWJSCs retained their short-fibroblast-like phenotype with no morphological changes when grown in MDA-TCM, TOV-TCM and controls (Fig. 1A, a–p). The hBMMSC controls (BM and CCD-FCM) retained their characteristic morphology of short flat fibroblastic-like cells (Fig. 1B, a–h). However, hBMMSCs exposed to MDA-TCM and TOV-TCM showed changes in morphology to thin long spindle-shaped cells typical of fibroblasts from day 10 to day 30 (Fig. 1B, i–p).

CELL PROLIFERATION: MTT ASSAY

The cell proliferation rates of the hWJSCs exposed to MDA-TCM and TOV-TCM decreased with time from day 10 to day 30 compared to hWJSCs grown in BM (Fig. 2A). The mean decreases in hWJSC proliferation when exposed to TOV-TCM and MDA-TCM were 24.64% and 21.40% on day 10; 30.56% and 23.09% on day 20; and 38.64% and 30.01% on day 30, respectively. These decreases observed for TOV-TCM and MDA-TCM were statistically significant when compared to controls.

The hBMMSC proliferation rates however increased in the presence of TOV-TCM and MDA-TCM from day 10 to day 30 compared to hBMMSCs grown in BM (Fig. 2B). The increases in hBMMSC proliferation when exposed to TOV-TCM and MDA-TCM were 32.86% and 23.56% on day 10; 55.01% and 50.76% on day 20; and 60.77% and 55.88% on day 30, respectively. These increases observed for TOV-TCM and MDA-TCM were statistically significantly when compared to the control.

CD MARKER ANALYSIS OF hWJSCs AND hBMMSCs

The hWJSCs and hBMMSCs expressed similar CD marker signature profiles in BM before exposure to tumor-conditioned medium [hWJSCs: CD44, CD29, CD13, and CD105 (97.69–99.95%) and CD14 (1.82%) (Fig. 3A, column 1); hBMMSCs: CD44, CD29, CD13, and CD105 (93.72–98.22%) and CD14 (4.76%)] (Fig. 3B, column 1). By day 30, these CD marker percentages for hWJSCs exposed to MDA-TCM and TOV-TCM did not change significantly (Fig. 3A, columns 3 and 4). However the percentages for CD44, CD29, CD13, and CD105 decreased significantly to 40.82–82.53% and 68.72–88.92%, respectively, for the hBMMSCs exposed to MDA-TCM and TOV-TCM (Fig. 3B, columns 3 and 4).

QUANTITATIVE REAL TIME POLYMERASE CHAIN REACTION (qRT-PCR)

When compared to hWJSCs, qRT-PCR analysis of hBMMSCs cultured in MDA-TCM and TOV-TCM showed high level expression of TAF-related genes [fibroblast specific protein (FSP), fibroblast activated protein (FAP), tenascin-C (Tn-C), thrombospondin-1 (Tsp-1), epidermal growth factor (EGF), basic fibroblast growth

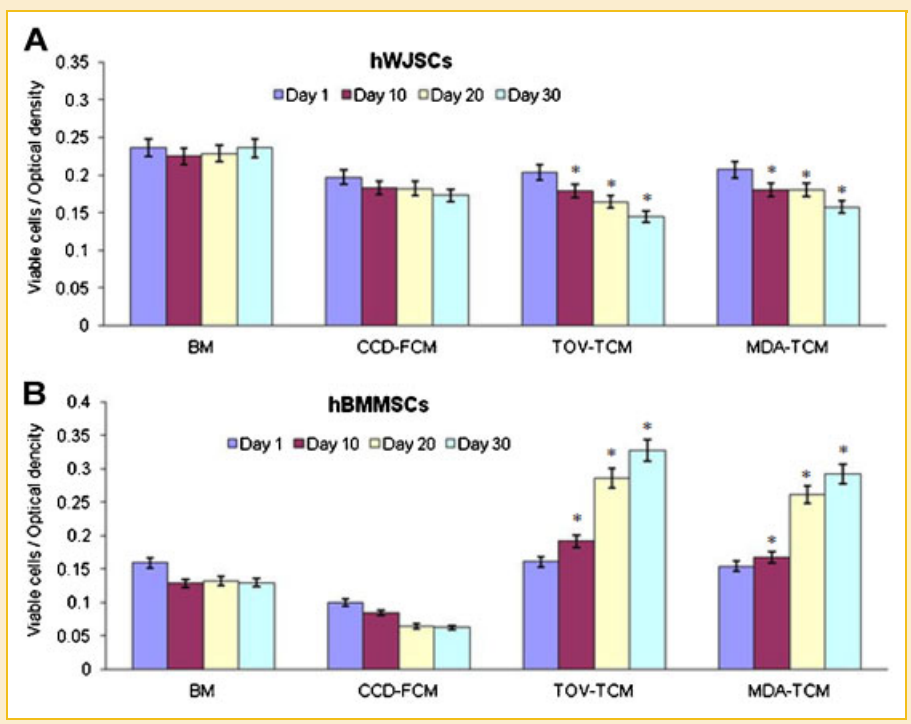

Fig. 2. Cell proliferation rates (MTT assay) of hWJSCs and hBMMSCs exposed to TOV-TCM, MDA-TCM and controls. A: The cell proliferation rates of hWJSCs exposed to MDA-TCM and TOV-TCM decreased from day 10 to day 30 compared to control (BM) whereas (B) the hBMMSC proliferation rates increased in the presence of TOV-TCM and MDA-TCM compared to controls. The values are expressed as mean ± SEM of three replicates and asterisks (*) indicate statistical significance of $P < 0.05$. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

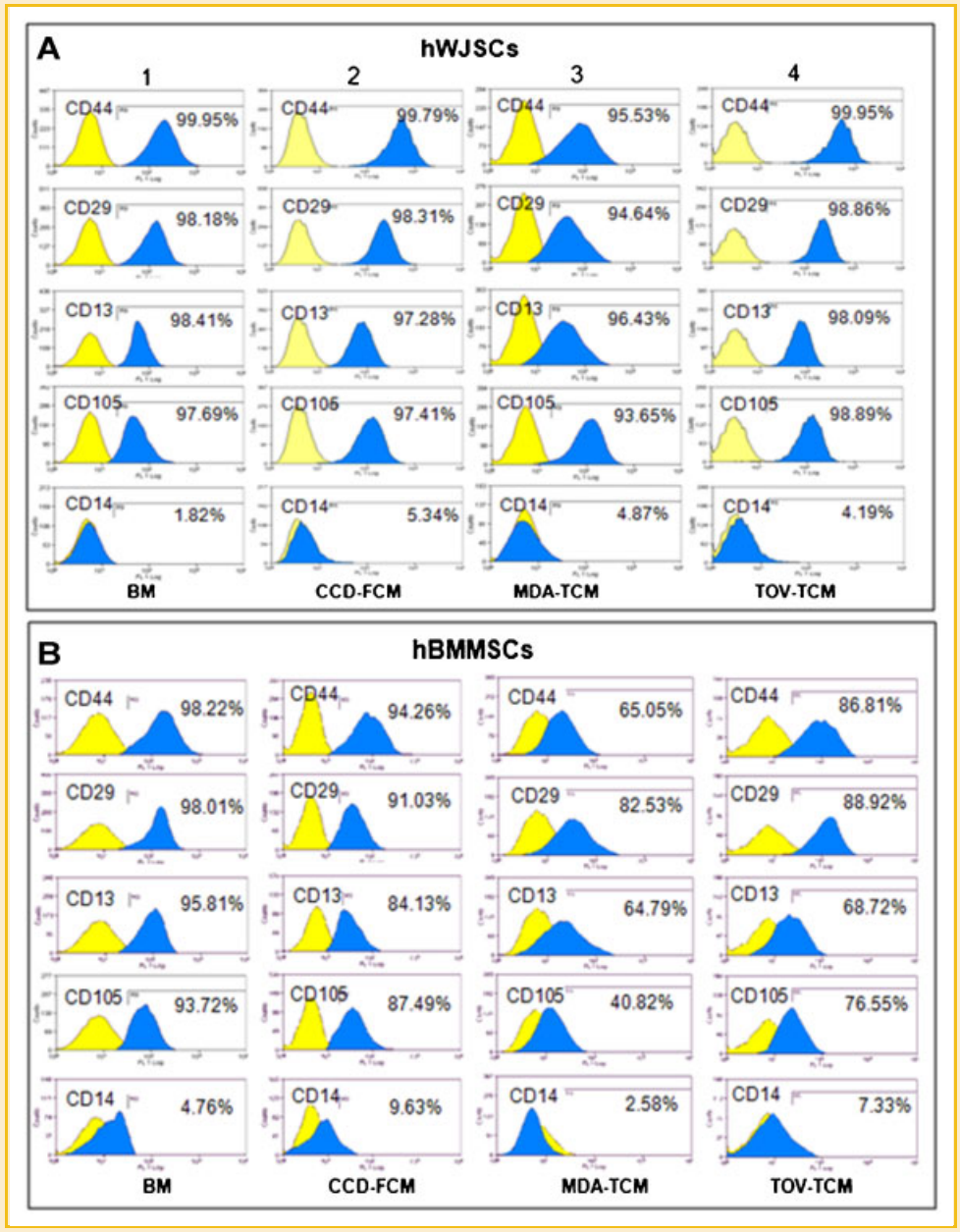

Fig. 3. Flow cytometry CD marker profiles of hWJSCs and hBMMSCs after exposure to MDA-TCM and TOV-TCM for 30 days. A,B: The hWJSCs and hBMMSCs expressed similar CD markers (CD44, CD29, CD13, CD105, CD14). After 30 days of exposure to MDA-TCM and TOV-TCM the CD marker percentages did not change for the hWJSCs but they all decreased for the hBMMSCs. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

factor (bFGF), interleukin-6 (IL-6), alpha-smooth muscle actin (α-SMA), and vascular endothelial growth factor (VEGF)] (Fig. 4). The fold increases in expression levels for all these genes compared to controls ranged from 0.23 to 92.18 and the fold increases for each gene were significantly greater than that of hWJSCs and controls.

IMMUNOCYTOCHEMISTRY ANALYSIS

The hBMMSCs cultured in MDA-TCM and TOV-TCM showed positive staining for the tumor-associated markers FSP, VEGF, EGF, and Tn-C (Fig. 5A, i–p) while the hWJSCs exposed to TOV-TCM and MDA-TCM were weakly positive for VEGF (Fig. 5A, e,f) and negative for FSP, EGF and Tn-C (Fig. 5A, a–d, g–h).

GROWTH FACTOR ANALYSIS

The mean ± SEM levels of interleukin 6 (IL-6), TGF-β and VEGF secreted by hWJSCs in TOV-TCM and MDA-TCM were either not significantly different or significantly lower when compared to controls (Fig. 5B,a) whereas the levels secreted by hBMMSCs in TOV-TCM and MDA-TCM were significantly greater than controls (Fig. 5B,b). The mean increases (pg/ml) for IL-6 were 11,180.95 and

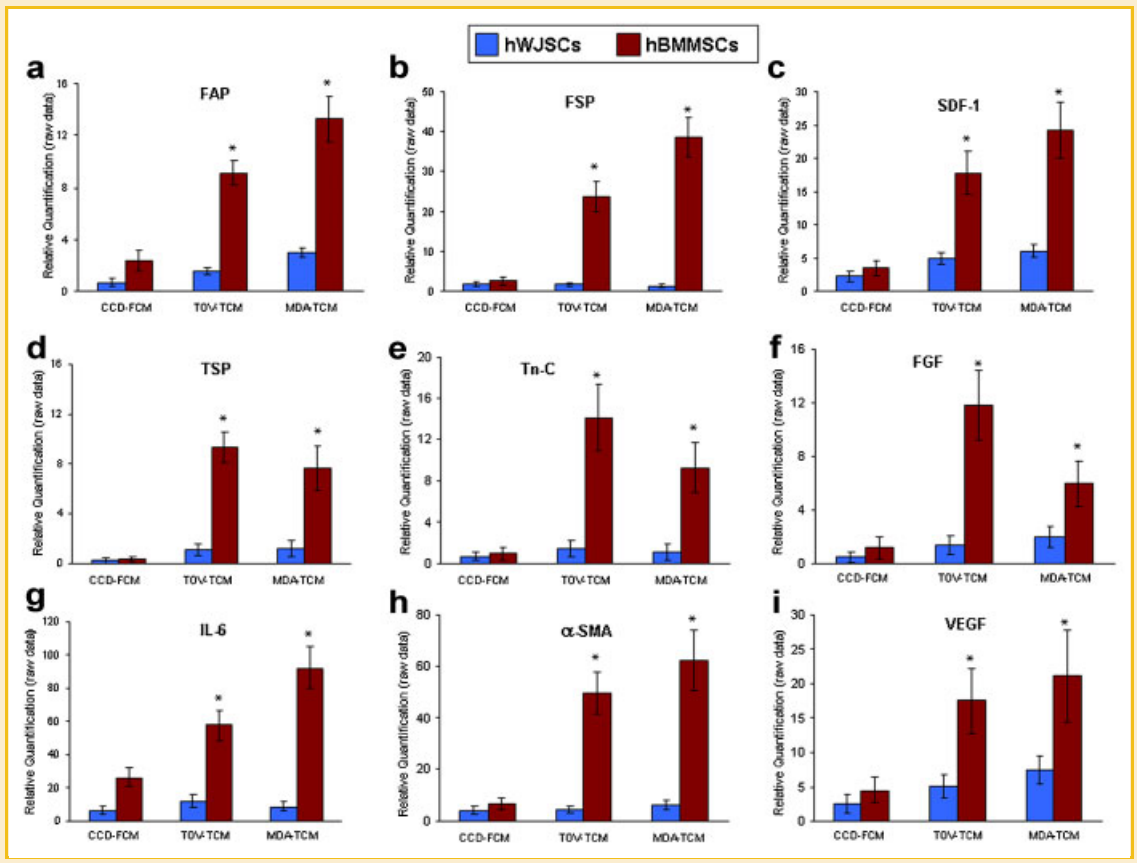

Fig. 4. Real-time RT-PCR of TAF-related genes. a–i: The hBMMSCs exposed to TOV-TCM and MDA-TCM showed significantly high expression of the TAF related genes (FAP, FSP, SDF-1, TSP, Tn-C, FGF, IL-6, α-SMA, and VEGF) compared to hWJSCs and controls. Asterisks (*) indicate >2-fold increases compared to controls. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

5,578.79, TGF-β (1,175.93 and 3,094.06) and VEGF (13,574.41 and 14,014.23) for TOV-TCM and MDA-TCM, respectively.

LUCIFERASE ACTIVITY (TUMOR CELL GROWTH ASSAY)

The luminescence levels of the luciferase-tagged MDA-MB-231 and TOV-112D cells that were cocultured with hWJSCs previously exposed to MDA-TCM and TOV-TCM, respectively, were not significantly different from the tagged MDA-MB-231 and TOV-112D cells that were not exposed to MDA-TCM and TOV-TCM previously (Fig. 6a,b). On the other hand, when luciferase-tagged MDA-MB-231 and TOV-112D cells were cocultured with hBMMSCs previously exposed to MDA-TCM and TOV-TCM respectively, the luminescence levels increased significantly compared to tagged MDA-MB-231 and TOV-112D cells cocultured with hBMMSCs not exposed to MDA-TCM and TOV-CM previously (Fig. 6c,d).

DISCUSSION

It has been shown conclusively by many workers that TAFs encouraged tumor cell growth of solid tumors compared to normal fibroblasts in non-tumor sites [Blankenstein, 2005; Mishra et al., 2008; Wels et al., 2008]. The differences however in the nature and properties of TAFs in comparison to normal fibroblasts have not been clearly delineated although it has been reported that breast carcinoma-associated TAFs promote breast carcinoma proliferation, angiogenesis and expression of characteristic myofibroblastic markers [Orimo et al., 2005]. Interestingly, several reports point to the fact that such TAFs originate from hematopoietic bone marrow precursor/stem cells [Ishii et al., 2003; Studeny et al., 2004; LaRue et al., 2006].

It was hypothesized that the actual precursor cell that transforms to TAF in solid tumors is the hBMMSC [Mishra et al., 2008]. The same workers confirmed this hypothesis by examining the phenotypic effect of a 30-day exposure of hBMMSCs to the secretions in conditioned medium produced by the human breast cancer cell line MDA-MB-231. Their results showed that the hBMMSCs differentiated into myofibroblasts that expressed α-SMA, vimentin, FSP and SDF-1 which are the typical markers for TAFs. They finally concluded that hBMMSCs migrate to the site of the tumor and when induced by tumor-derived factors transform into TAFs that become part of the tumor environment with the TAFs subsequently enhancing angiogenesis and further growth of the tumor.

Our objective in the present study was to examine whether TAF formation was exclusive to MSCs of the bone marrow or was also a

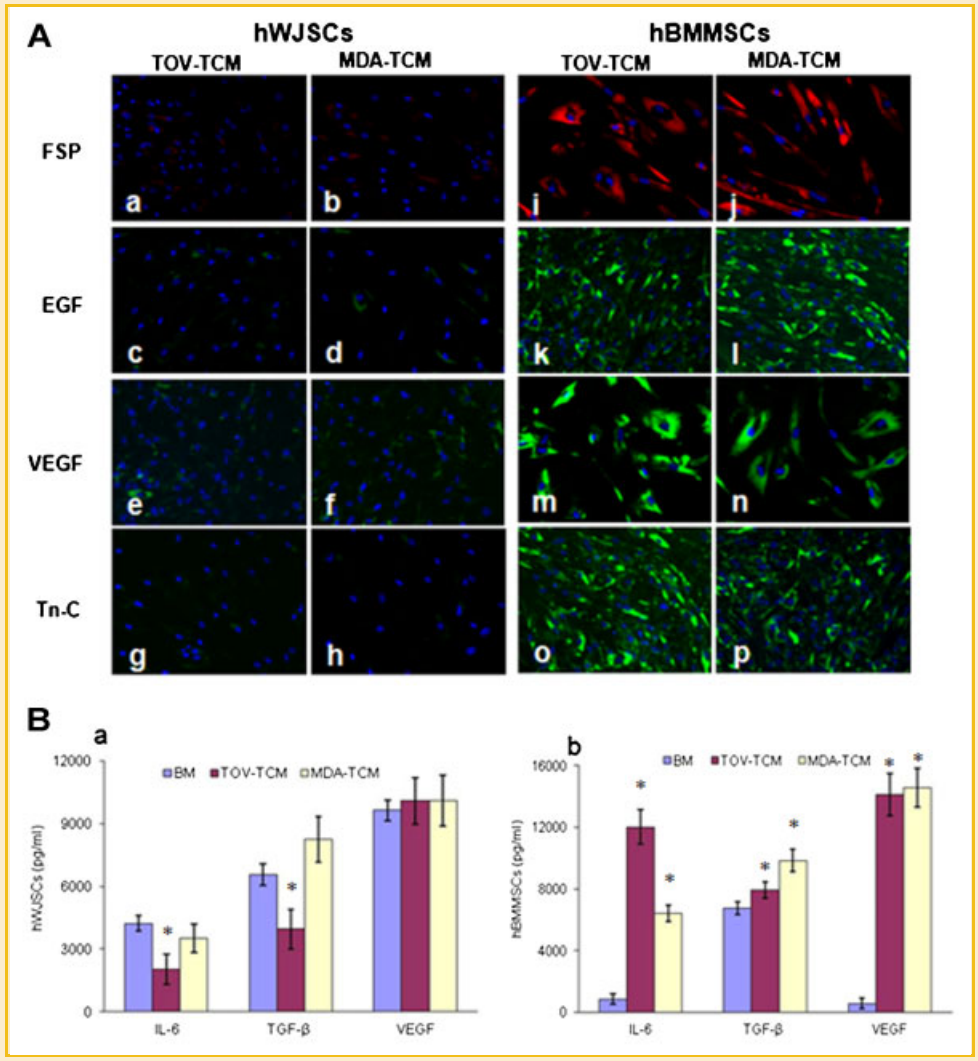

Fig. 5. Tumor-associated markers (immunocytochemistry) and growth factor analysis of hWJSCs and hBMMSCs exposed to TOV-TCM and MDA-TCM. A: The hBMMSCs cultured in TOV-TCM and MDA-TCM showed positive staining for the tumor-associated markers FSP, VEGF, EGF, and Tn-C (i–p) while the hWJSCs exposed to TOV-TCM and MDA-TCM were weakly positive for VEGF (e,f) and negative for FSP, EGF and Tn-C (a–d, g–h). B: The levels of IL-6, TGF-β, and VEGF secreted by hWJSCs in TOV-TCM and MDA-TCM were not significantly different from controls whereas the levels secreted by hBMMSCs in TOV-TCM and MDA-TCM were significantly greater than controls. Asterisks (*) indicate statistical significance of $P < 0.05$. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

property of other MSCs. We chose the human Wharton's jelly MSC (hWJSC) as the alternative MSC to examine this hypothesis based on the fact that even though hWJSCs and hBMMSCs have a common early embryonic origin and are both typically MSCs when characterized, there are still distinct differences between them [Troyer and Weiss, 2008; Fong et al., 2010a].

hWJSCs are a defined homogeneous stem cell population lying in the gelatinous matrix of the Wharton's jelly which is located in between the umbilical blood vessels of the umbilical cord. The actual origin and future potential of these hWJSCs for clinical application in the human were recently reviewed by Taghizadeh et al. [2011]. In early human embryonic development hematopoiesis takes place first in the yolk sac and later in the aorta-gonad-mesonephros (AGM) region. It was reported in some elegant studies by Wang et al. [2008] that hematopoietic cells and MSCs from the yolk sac and AGM migrated through the umbilical cord to the placenta during early human development between days 4 and 12. From the placenta these cells showed a second wave of migration back again via the umbilical cord to the fetal liver and then finally homed in the fetal bone marrow. In these migrating colonies were primitive HSCs and MSCs and during their migration to and fro via the placenta and umbilical cord some of the MSCs became trapped in the gelatinous Wharton's jelly and remained there throughout gestation [Wang et al., 2008]. These MSCs then multiplied to finally become the defined homogeneous hWJSC population observed in the Wharton's jelly.

Therefore, given their common origin, the nature and properties of hWJSCs and hBMMSCs would be expected to be similar but interestingly distinct differences exist between them probably brought about by the microenvironments in their new locations. For example, compared to hBMMSCs, hWJSCs are highly proliferative, maintain their stemness properties for prolonged periods of time in

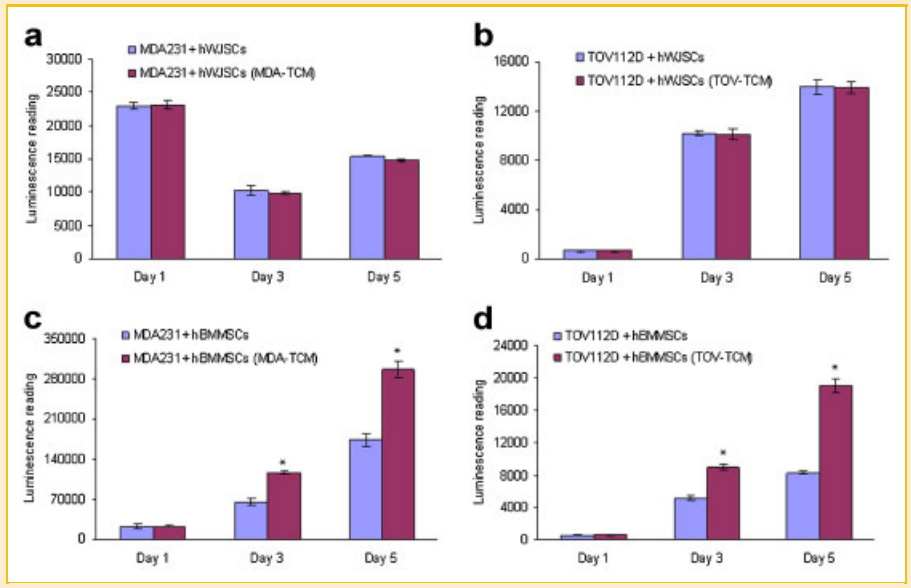

Fig. 6. Luminescence levels when luciferase-tagged MDA231 and TOV112D cells were cocultured with hWJSCs and hBMMSCs previously exposed to MDA-TCM and TOV-TCM. a,b: By day 5, the luminescence levels of the tagged MDA231 and TOV112D cells cocultured with hWJSCs previously exposed to MDA-TCM and TOV-TCM were not significantly different from the tagged MDA231 and TOV112D cells cocultured with hWJSCs and not exposed to MDA-TCM and TOV-TCM. c,d: However, by day 5, luminescence levels of tagged MDA231 and TOV112D cells cocultured with hBMMSCs previously exposed to MDA-TCM and TOV-TCM increased significantly compared to tagged MDA231 and TOV112D cells cocultured with hBMMSCs and not exposed to MDA-TCM and TOV-CM. Asterisks (*) indicate statistical significance of $P < 0.05$. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

culture, possess a slightly different CD marker signature [Fong et al., 2007, 2010a; Karahuseyinglu et al., 2007], are hypoimmunogenic [Weiss et al., 2008; Fan et al., 2011], non-tumorigenic [Gauthaman et al., 2011] and more interestingly shown to possess anti-tumorigenic properties [Ayuzawa et al., 2009; Ganta et al., 2009].

These unique differences between hWJSCs and hBMMSCs are probably reflected in the results of the present study where it was observed that hWJSCs behaved differently from hBMMSCs when exposed to the secretory factors of the TCM of two cancer cell lines [breast carcinoma (MDA-MB-231) and ovarian carcinoma (TOV-112D)]. The MTT assay, immunohistochemistry, MSC-CD profile, growth factor analysis and luciferase assays all showed that in the presence of TCM of these two cancer cell lines hWJSCs did not transform into TAFs while hBMMSCs did, and the fold increases in the biochemical markers for TAFs (FAP, FSP, SDF-1, TSP, TN-c, FGF, IL-6, α-SMA, and VEGF) in the hBMMSCs were significantly higher than the hWJSCs and controls. The transformation of hBMMSCs to TAFs in the presence of TCM of MDA231 cells is consistent with other studies [Mishra et al., 2008; Spaeth et al., 2009]. The transformation of hBMMSCs to TAFs in the presence of TCM of another different cancer cell line (TOV-112D) suggests that this phenomenon is not confined only to breast cancer. This is consistent with the studies of Spaeth et al. [2009] who showed that hBMMSCs acquired a TAF phenotype following systemic recruitment into adenocarcinoma xenograft models for ovarian, breast, and pancreatic cancers.

TAFs were shown to promote tumor growth and metastasis by modulating the tumor immune microenvironment in a murine breast cancer model [Liao et al., 2009] and SDF-1 was shown to be an important player in promoting such tumor growth and angiogenesis [Orimo et al., 2005]. Recently, Mishra et al. [2011] suggested that there was cross-talk between TAFs and cancer cells and that this was brought about mainly by the CXCL12 (SDF-1)-CXCR4 pair of chemokines. Thus, the low level expression of SDF-1 in the hWJSCs in the present study after they were exposed to TCM of both cancer cell lines confirms the non-transformation of the hWJSCs to the TAF phenotype.

The reports of Ganta et al. [2009], Ayuzawa et al. [2009], and our unpublished studies (Gauthaman et al., unpublished work) showed that hWJSCs possess anti-tumorigenic properties. Also, thus far, there have been no reports of tumorigenesis induced by hWJSCs when injected into diseased animal models. Additionally, a recent report by our group showed that when naïve hWJSCs were injected into immunodeficient SCID mice via several routes, none of the animals produced tumors after 20 weeks after injection compared to a parallel batch of SCID mice that developed tumors after 6 weeks when human embryonic stem cells (hESCs) were injected via similar routes [Gauthaman et al., 2011]. Bone marrow MSCs and neural MSCs however were shown to be linked to osteosarcomas and brain tumors, respectively, when transplanted in animals and humans [Amariglio et al., 2009; Mohseny et al., 2009]. Recently, allogeneic bone marrow-derived HSCs were shown to contribute to oral squamous cell carcinoma in a patient after HSC transplantation [Hu et al., 2011]. It thus appears that hWJSCs are a safe MSC for future clinical application because they appear to possess the unique properties of being anti-tumorigenic, non-tumorigenic and do not transform to the TAF phenotype that is associated with enhanced growth of solid tumors.

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