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# Human Umbilical Cord Wharton's Jelly Stem Cell (hWJSC) Extracts Inhibit Cancer Cell Growth In Vitro

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## ABSTRACT

Umbilical cord mesenchymal stem cells (MSCs) have been shown to inhibit breast cancer cell growth but it is not known whether this effect is specific to only breast cancer cells. We compared the effects of human Wharton's jelly stem cell (hWJSC) extracts [conditioned medium (hWJSC-CM) and cell lysate (hWJSC-CL)] on breast adenocarcinoma (MDA-MB-231), ovarian carcinoma (TOV-112D), and osteosarcoma (MG-63) cells. The cells were treated with either hWJSC-CM (50%) or hWJSC-CL (15 μg/ml) for 48–72 h and changes in cell morphology, proliferation, cycle, gene expression, migration, and cell death studied. All three cancer cell lines showed cell shrinkage, blebbing, and vacuolations with hWJSC-CL and hWJSC-CM compared to controls. MTT and BrdU assays showed inhibition of cell growth by 2–6% and 30–60%, while Transwell migration assay showed inhibition by 20–26% and 31–46% for hWJSC-CM and hWJSC-CL, respectively, for all three cancer cell lines. Cell cycle assays showed increases in sub-G1 and G2/M phases for all three cancer cell lines suggestive of apoptosis and metaphase arrest. AnnexinV-FITC and TUNEL positive cells seen in TOV-112D and MDA-MB-231 suggested that inhibition was via apoptosis while the presence of anti-BECLIN1 and anti-LC3B antibodies seen with MG-63 indicated autophagy. Upregulation of pro-apoptotic BAX and downregulation of anti-apoptotic BCL2 and SURVIVIN genes were observed in all three cancer cell lines and additionally the autophagy genes (ATG5, ATG7, and BECLIN1) were upregulated in MG-63 cells. hWJSCs possess tumor inhibitory properties that are not specific to breast cancer cells alone and these effects are mediated via agents in its extracts. J. Cell. Biochem. 113: 2027–2039, 2012. 

**KEY WORDS:** APOPTOSIS; AUTOPHAGY; CANCER CELLS; CELL INHIBITION; HUMAN WHARTON'S JELLY STEM CELL CONDITIONED MEDIUM AND LYSATE

Treatment of cancer continues to be a major challenge and cancer remains a leading cause of death world-wide [Jemal et al., 2011]. The poor outcome in the therapeutic management of various types of cancers is partly due to their aggressiveness, metastatic potential, the prevailing internal milieu, and the host defense mechanism. Understanding the molecular mechanisms and signaling pathways involved in oncogenesis has led to the development of novel targeted anticancer therapies [Bhardwaj et al., 2010; Yuan et al., 2011]. These agents target cellular events, such as proliferation, cell cycle progression, apoptosis, migration, invasion, and angiogenesis. Some of these agents act as inhibitors of the receptors of vascular endothelial growth factor (VEGF), epidermal growth factor (EGFR), and matrix metalloproteinases (MMPs), while others act as promoters of apoptosis or modulators of tyrosine kinase/mammalian targets of rapamycin (mTOR) signaling pathways [Rosen et al., 2010].

In recent years, it has been shown that mesenchymal stem cells (MSCs) residing within tissue specific niches in vivo are recruited to sites of tissue damage and inflammation [Chamberlain et al., 2007]. This property has led to the use of MSCs as vehicles for gene therapy and to deliver anticancer agents, such as cytokines, apoptotic inducers, interferons, and pro-drugs to tumor sites for growth inhibition [Fritz and Jorgensen, 2008; Prockop, 2009]. Apart from acting as delivery vehicles, MSCs have also been shown to interact with tumors and cause tumor growth inhibition and abolishment depending upon the tumor type they come into contact with. Khakoo et al. [2006] demonstrated that human bone marrow MSCs (hBMMSCs) injected intravenously in an in vivo model of Kaposi's

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sarcoma homed to the tumor site tissue and inhibited tumor growth. It was later shown that intra-tumoral injection of rat umbilical cord matrix stem cells (rUCMSCs) caused complete regression of rat mammary carcinomas with no evidence of tumor metastasis [Ganta et al., 2009]. Similarly, when unengineered human UCMSCs (hUCMSCs) were injected intravenously in a xenotransplant rat model in which human breast carcinoma (MDA-MB-231) was induced, the hUCMSCs homed into the lung metastases brought about by the breast carcinoma and reduced tumor burden [Ayuzawa et al., 2009]. More recently, hUCMSCs were shown to inhibit breast cancer cell growth (MDA-MB-231) in a SCID mouse model through secretion of dickkopf and suppression of the Wnt pathway [Sun et al., 2010].

In all these studies using hUCMSCs the exact origin of stem cells in the human umbilical cord that exert such anticancer effects is not defined given the fact that MSCs have been isolated from various compartments within the human umbilical cord, viz., amnion [Illancheran et al., 2007], subamnion [Kita et al., 2010], perivascular areas surrounding the umbilical blood vessels [Sarugaser et al., 2005], and Wharton's jelly [Weiss et al., 2006; Fong et al., 2007]. Additionally, whether the anticancer effect is specific to only certain cancers and whether the mechanism of action is mediated via cell-to-cell contact or through the secretions of the hUCMSCs are not known.

We have derived homogeneous populations of MSCs specifically from the gelatinous Wharton's jelly (hWJSCs) of the human umbilical cord [Fong et al., 2007, 2010a]. These hWJSCs when characterized are unique in that they have their own MSC-CD signature, have prolonged stemness properties in vitro (up to 10 passages), can be differentiated into many desirable tissues, are hypoimmunogenic and do not induce tumorigenesis in immunodeficient mice [Gauthaman et al., 2010; Fong et al., 2010a, 2011a; Gauthaman et al., 2011]. Comparative microarray transcriptome profile analysis showed that hWJSCs also have a unique transcriptome profile in that they showed high level expression of pro-apoptotic and tumor suppressor genes which were not observed in pluripotent embryonic stem cells, other MSC types, and cancer cells [Fong et al., 2010b].

These unique properties of hWJSCs prompted us to explore the anticancer properties of its extracts [cell lysate (hWJSC-CL) and conditioned medium (hWJSC-CM)], on three human cancer cell lines [ovarian adenocarcinoma (TOV-112D), osteosarcoma (MG-63), and breast adenocarcinoma (MDA-MB-231)] in vitro.

## MATERIALS AND METHODS

### CELL CULTURE

**Human Wharton's jelly stem cells.** Human Wharton's jelly stem cells (hWJSCs) were derived and propagated according to our earlier published protocols [Fong et al., 2007, 2010a]. Umbilical cords were donated for this study after informed patient consent and ethical approval from the Ministry of Health, Institutional Domain Specific Review Board (DSRB), Singapore. Briefly, 15–30 cm long pieces of umbilical cord were submitted to the laboratory in Hank's balanced salt solution (HBSS; Invitrogen Life Technologies, Carlsbad, CA) in sterile plastic containers. Each piece was cut further into smaller 3 cm pieces and each smaller piece cut open and its inner surface placed face down in a sterile plastic Petri dish (100 mm; Becton Dickinson, BD, Franklin Lakes, NJ) containing 1.5 ml of an enzymatic solution so that only the Wharton's jelly came into contact with the enzymes. The enzymatic solution comprised a mixture of collagenase types I and IV and hyaluronidase (Sigma Chemical Co, MO) in DMEM medium (Invitrogen). The Petri dishes were incubated for 45 min at 37°C in a 5% $CO_2$ in air atmosphere. The enzymatic solution containing the gelatinous Wharton's jelly was then syringed through a 18 G needle to break up the gelatinous masses and release the hWJSCs. The cell suspension was then centrifuged at 300*g* for 10 min, supernatant discarded and cell pellets resuspended in hWJSC culture medium that comprised 80% DMEM medium supplemented with 20% fetal bovine serum, 16 ng/ml basic fibroblast growth factor (Millipore Bioscience research agents, Temecula, CA), 1% non-essential amino acids, 2 mM L-glutamine, 0.1 mM β-mercaptoethanol, 1% insulin–transferrin–selenium and antibiotic mixture of penicillin (50 IU), and streptomycin (50 μg/ml; Invitrogen). The hWJSCs were seeded in T25 tissue culture flasks (BD) and cultured at 37°C in a 5% $CO_2$ in air atmosphere.

**Human cancer cells and foreskin fibroblasts.** The human cancer and non-cancer cells (controls) used for this study were commercial cell lines purchased from the American Type Culture Collection (ATCC, Rockville, MD) and included ovarian adenocarcinoma (TOV-112D), osteosarcoma (MG-63), breast adenocarcinoma (MDA-MB-231), and human foreskin fibroblasts (CCD-1112 sk). Ethical approval for use of these commercial human cell lines was given by the National University of Singapore (NUS) Institutional Review Board (NUS-IRB). The human cancer cell lines (TOV-112D, MG-63, MDA-MB-231) and fibroblast cell line (CCD-1112 sk) were initially cultured in their respective culture media after thawing as specified by ATCC, and were then weaned and cultured from the first passage onwards in hWJSC medium for a minimum of three passages (3P) before being used for the experiments in this study. The cancer cell lines and the control fibroblast cell line had similar proliferation rates as that of hWJSCs.

### PREPARATION OF hWJSC EXTRACTS

Human Wharton's jelly stem cell lysate was prepared from early passages of hWJSCs (P3–P7) that were grown to 80% confluence using a commercial mammalian cell extraction kit containing a protease inhibitor cocktail and dithiothreitol (BioVision, Mountain View, CA). Briefly, the hWJSC monolayers were washed once with phosphate-buffered saline that contained no calcium and magnesium, that is, $PBS^{(-)}$, then dissociated with trypsin-EDTA (TrypLE™ express, Invitrogen) and centrifuged at 500*g* for 5 min to obtain a cell pellet. The pellet was resuspended in 100 μl of cell lysis buffer and resuspended several times and then incubated on ice for 45 min. The contents were then centrifuged using high speed (12,000*g* for 15 min) and the clear supernatant (hWJSC-CL) was separated and stored at −80°C until use. The total protein content was measured using a Nanodrop™ spectrophotometer (Nanodrop Technologies, Wilmington, DW). hWJSC-CL containing 15 μg/ml protein was used for all experiments.

Human Wharton's jelly stem cell conditioned medium was prepared by growing confluent hWJSC (P3-P7) monolayers (80% confluence) in hWJSC medium and then separation of the medium after 48 h. The medium was filter-sterilized using a 0.22 μm Millex-GP syringe filter (Millipore, Billerica, MA) and the pH and osmolality of the hWJSC-CM optimized before use. The pH and osmolality of the neat hWJSC-CM collected at 48 h from 80% confluent early passages (P4–P8) of hWJSCs ranged from $7.68 \pm 0.12$ to $8.01 \pm 0.02$ and $326.67 \pm 0.67$ to $335.33 \pm 0.88$, respectively. To make various concentrations of hWJSC-CM (50%, 75%, and 100%), the neat 100% hWJSC-CM was diluted accordingly in freshly prepared hWJSC medium.

### EXPOSURE OF CANCER CELLS TO hWJSC-CM AND hWJSC-CL

The three cancer cell types (ovarian, breast, and osteosarcoma) and the controls (hWJSCs and foreskin fibroblasts) were exposed to various concentrations of hWJSC-CM (50%, 75%, and 100%) and hWJSC-CL (15 μg/ml protein) for the various evaluations [morphology, cell viability and proliferation, cell cycle analysis, annexin-V FITC assay, cell migration assay, TUNEL assay, immunohistochemistry, and gene expression (qRT-PCR)].

### CELL MORPHOLOGY

The human cancer cells (TOV-112D, MG-63, and MDA-MB-231) and the controls [human foreskin fibroblast cells (CCD-1112 sk) and hWJSCs] were cultured in two sets of 24-well tissue culture plates for the two treatments (hWJSC-CM and hWJSC-CL; BD) at a seeding density of $2 \times 10^4$ cells/well. After overnight culture the medium was replaced with hWJSC-CM (50%) for one set and hWJSC-CL (15 μg/ml protein) for the other. Fresh changes of the hWJSC-CM and hWJSC-CL were provided to the wells at 48 h and exposure of the cells to hWJSC-CM and hWJSC-CL monitored. The changes in cell morphology were recorded daily and photographed using inverted phase contrast optics (Nikon Instruments, Tokyo, Japan).

### CELL VIABILITY AND PROLIFERATION

**MTT assay.** The cell viability rates of the cancer cell lines (TOV-112D, MG-63, MDA-MB-231) and the controls (CCD-1112 sk, hWJSCs) were evaluated using the MTT assay. The cells were cultured at a seeding density of $2 \times 10^4$ cells/well in 24-well tissue culture plates in either hWJSC-CM (50%, 75%, and 100%) or hWJSC-CL (5, 10, and 15 μg/ml protein) for 72 h with fresh changes of the hWJSC-CM and hWJSC-CL at 48 h. The cell viability assay was performed using a MTT reagent kit [3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide] (Sigma, St. Louis, MO) according to the manufacturer's instructions. Briefly, 10 μl MTT reagent (final concentration of 0.5 mg/ml) was added to the culture dishes and incubated for 4 h until a purple precipitate was visible. The supernatant was then aspirated and 100 μl of the detergent reagent was added and incubated in the dark for 2 h. Absorbance at 570 nm was spectrophotometrically measured using a microplate ELISA reader (μQuant-BioTek, Winooski, VT) with a reference wavelength of 630 nm. Following deduction of the blank cell absorbance, the treated cell absorbance over the control cell absorbance was expressed as a percentage of cell survival or proliferation.

**BrdU assay.** The incorporation of 5-bromo-2′-deoxyuridine (BrdU) into the cellular DNA during cell proliferation was assessed in the cancer and control cells using the BrdU assay kit (Cell Signaling Technology, Danvers, MA). The cancer and control cells were cultured at a seeding density of $2 \times 10^3$ cells in 96-well tissue culture plates (NUNC, Rochester, NY) in 100 μl of either hWJSC-CM (50%) or hWJSC-CL (15 μg/ml protein) for 72 h with fresh changes of the hWJSC-CM and hWJSC-CL at 48 h. The BrdU assay was performed according to the manufacturer's instructions. Briefly, 10 μM of BrdU solution was added to each well and the cells incubated for 30 min at 37°C in a 5% $CO_2$ in air atmosphere after which the medium was removed and the cells incubated for a further 30 min with the fixing/denaturing solution provided with the kit. Subsequently, the plate was incubated for 1 h with BrdU detection antibody solution followed by three washes with the wash buffer. The HRP-conjugate solution was then added and incubated for 30 min followed by similar washing steps and incubation with the substrate solution for 30 min. The enzymatic reaction was finally stopped and the reaction products were quantified by measuring the absorbance at 450 nm using a microplate ELISA reader (μQuant-BioTek, Winooski, VT).

### CELL CYCLE ANALYSIS

The cancer and control cells were cultured at a seeding density of $2 \times 10^5$ cells in T25 tissue culture flasks (BD) in hWJSC-CM (50%) and in hWJSC-CL (15 μg/ml protein) for 72 h with fresh changes of the respective media at 48 h. The monolayers were then detached and disassociated with trypsin-EDTA (Invitrogen), washed and fixed in ice-cold 70% ethanol. The fixed cells were stained with 50 μg/ml propidium iodide (PI) in PBS containing 0.1% Triton X-100 and 50 μg/ml RNAse-A, and then analyzed using a flow cytometer (Epics-Altra, Beckman Coulter) and Summit 4.3 (Beckman Coulter).

### ANNEXIN V-FITC ASSAY

The cancer and control cells were cultured at a seeding density of $2 \times 10^5$ cells in T25 tissue culture flasks (BD) in hWJSC-CM (50%) and in hWJSC-CL (15 μg/ml protein) for 48 h. The cells were then disassociated with trypsin-EDTA (Invitrogen), washed once with $PBS^{(-)}$ and then washed with Annexin V binding buffer (1×). The cells were stained with 5 μl Annexin V-FITC at room temperature for 15 min and then counterstained with PI (1 μg/ml) and finally analyzed using a CyAn™ ADP Analyzer and Summit 4.3 (Beckman Coulter).

### CELL MIGRATION ASSAY

The cancer and control cells were first cultured in serum-free medium (Invitrogen) for 24 h. The serum-starved cells were then seeded at a density of $1 \times 10^5$ cells/well and cultured in the upper chamber of 24-well transwell (8 μm pore size) culture plates (Corning Costar Corporation, Cambridge, MA) in serum-free medium. Two hundred fifty milliliters of either hWJSC-CM (50%) or hWJSC-CL (15 μg/ml protein) was added to the lower chamber of wells and cell migration from upper to lower chambers was evaluated after 24 h. The culture inserts were treated with the cell dissociation buffer in new wells of 24-well plates ensuring that all the cells attached to the lower surface were completely dislodged

into the new well. The migrated cells were quantified by measuring their absorbance at 570 nm following treatment with MTT reagent.

### TdT (TERMINAL DEOXYNUCLEOTIDYL TRANSFERASE)-MEDIATED dUDP NICK-END LABELING (TUNEL) ASSAY

The Dead End Fluorometric System (Promega) was used according to the manufacturer's instructions to detect TUNEL positive cells. The cancer and control cells were cultured at a seeding density of $2 \times 10^3$ cells/well in a 96-well tissue culture plate (NUNC, Rochester, NY) in hWJSC-CM (50%) and hWJSC-CL for 48 h. The cells were washed and fixed with 4% methanol-free formaldehyde solution for 25 min at 4°C followed by addition of Triton X-100 solution for 5 min at 4°C. Labeling of DNA was done by treating the cells with fluorescin-12dUTP and terminal deoxynucleotidyltransferase for 1 h at 37°C in a humidified chamber in the dark. The reaction was stopped by the addition of sodium chloride–sodium citrate solution, the cells washed thrice, and then stained with PI (1 μg/ml) in PBS containing 250 μg/ml of DNase-free RNase for 15 min at room temperature in the dark. The TUNEL positive cells were analyzed under a fluorescence microscope.

### IMMUNOHISTOCHEMISTRY

The osteosarcoma cells (MG-63) at a seeding density of $2 \times 10^4$ cells/well were separately cultured in a 24-well tissue culture plates (BD) in hWJSC-CM (50%) and hWJSC-CL (15 μg/ml protein) for 72 h and then analyzed for Beclin-1 and LC3B using immunohistochemistry. Briefly, the cells were fixed with ice-cold ethanol, washed with PBS, and treated with 10% normal goat serum (NGS). The cells were then incubated with rabbit polyclonal anti-Beclin-1 and anti-LC3B primary antibodies (1:100; Cell Signaling, Boston, MA) for 1 h and goat anti-rabbit secondary antibody (Alexa Fluor 588; 1:500 dilution) for 30 min. Following PBS washes, the cells were incubated with 0.5 μg/ml of 4′-6-diamidino-2-phenylindole (DAPI; Molecular probes, Invitrogen Life Technologies, Carlsbad, CA) for 5 min at room temperature, washed with PBS, and analyzed using fluorescence microscopy.

### QUANTITATIVE REAL-TIME POLYMERASE CHAIN REACTION (qRT-PCR)

The cancer and control cells were cultured at a seeding density of $2 \times 10^5$ cells in T25 tissue culture flasks (BD) in hWJSC-CM (50%) and in hWJSC-CL (15 μg/ml protein) for 48 h. Total RNA was extracted from the cells using TRIzol™ reagent (Invitrogen). RNA quality and quantity were measured using a Nanodrop™ spectrophotometer (Nanodrop technologies, Wilmington, DW) and all samples were treated with DNase-I prior to first-strand cDNA synthesis with random hexamers using the SuperScript™ first-strand synthesis system (Invitrogen). Primer sequences were taken from earlier published studies and were as follows. BAX: 5′-TGCTTCAGGGTTTCATCCAG-3′ (forward), 5′-GGCGGCAATCATCCTCTG-3′ (reverse); BCL2: 5′-GGCTGGGATGCCTTTGTG-3′(forward), 5′-CAGCCAGGAGAAATCAAACAGA-3′(reverse); SURVIVIN: 5′-ACCAGGTGAGAAGTGAGGGA-3′(forward), 5′-AACAGTAGAGGAGCCAGGGA-3′(reverse); ATG5: 5′-TGATCCTGAAGATGGGGAAA-3′ (forward), 5′-TCCGGGTAGCTCAGATGTTC-3′ (reverse); ATG7: 5′-CGGCGGCAAGAAATAATG-3′ (forward), 5′-CCCAACATCCAAGGCACTAC-3′ (reverse); BECLIN1: 5′-GAGGGATGGAAGGGTCTA-3′ (forward), 5′-GCCTGGGCTGTGGTAAGT-3′ (reverse); GAPDH: 5′-GCACCGTCAAGGCTGAGAAC-3′ (forward), 5′-GGATCTCGCTCCTGGAAGATG-3′ (reverse). qRT-PCR analysis was performed using the ABI PRISM 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA) using SYBR green as previously described and relative quantification was performed using the comparative $C_t$ $(2 - \Delta\Delta C_t)$ method.

### STATISTICAL ANALYSIS

Statistically significant differences between the cancer and control cells for the various assays were compared and analyzed using either one-way ANOVA with Bonferroni's multiple comparisons post hoc analysis or Student's *t*-test using the statistical package for Social Sciences (SPSS 13). The results were expressed as mean ± SEM from three different replicates for individual assays and a value of $P < 0.05$ was considered to be statistically significant.

## RESULTS

### CELL MORPHOLOGY

The three different cancer cell types (TOV-112D, MG-63, and MDA-MB-231) and the control fibroblast cells (CCD-1112 sk) maintained their characteristic morphology and grew well in hWJSC medium forming confluent monolayers in culture. After the hWJSC medium was changed to hWJSC-CM or hWJSC-CL and the cells grown for 72 h, the controls (CCD-1112 sk and hWJSCs) continued to maintain their typical morphology in culture.

However, the cancer cells (TOV-112D, MG-63, and MDA-MB-231) in hWJSC-CM showed decreases in cell numbers and production of degenerating/dead cells after 72 h compared to controls (Fig. 1). When exposed to hWJSC-CL, all three cancer cell lines also showed cell death with changes in their cell morphologies compared to controls. TOV-112D cells showed fragmentation, cell shrinkage, and numerous granular particles, while MDA-MB-231 showed varying degrees of membrane damage, blebbing, and cell debris. Unlike TOV-112D and MDA-MB-231, MG-63 showed different morphological changes in the form of numerous vacuolations in 50–60% of the cells (Fig. 1).

### CELL PROLIFERATION

**MTT assay.** The control cells (CCD-1112 sk and hWJSCs) cultured in hWJSC-CM and hWJSC-CL for 72 h grew well with no inhibition of proliferation in culture.

All three cancer cell lines (TOV-112D, MG-63, and MDA-MB-231) cultured in hWJSC-CM (50%, 75%, and 100%) showed decreases in cell proliferation. In Figure 2A, when compared within each cancer cell group, TOV-112D was inhibited by 2.05%, 3.44%, and 8.67%; MG-63 by 3.28%, 5.01%, and 13.06% and MDA-MB-231 by 16.39%, 20.73%, and 22.96% for 50%, 75%, and 100% hWJSC-CM, respectively, compared to their respective controls. Only the decreases in cell proliferation observed for MG-63 in hWJSC-CM (100%) and for MDA-MB-231 in hWJSC-CM (50%, 75%, and 100%) were statistically significant. When compared with the hWJSC group, TOV-112D was increased by 8.16%, 6.62%, and 7.03%; MG-63 was inhibited by 8.79%, 10.07%, and 5.24%; and MDA-MB-231

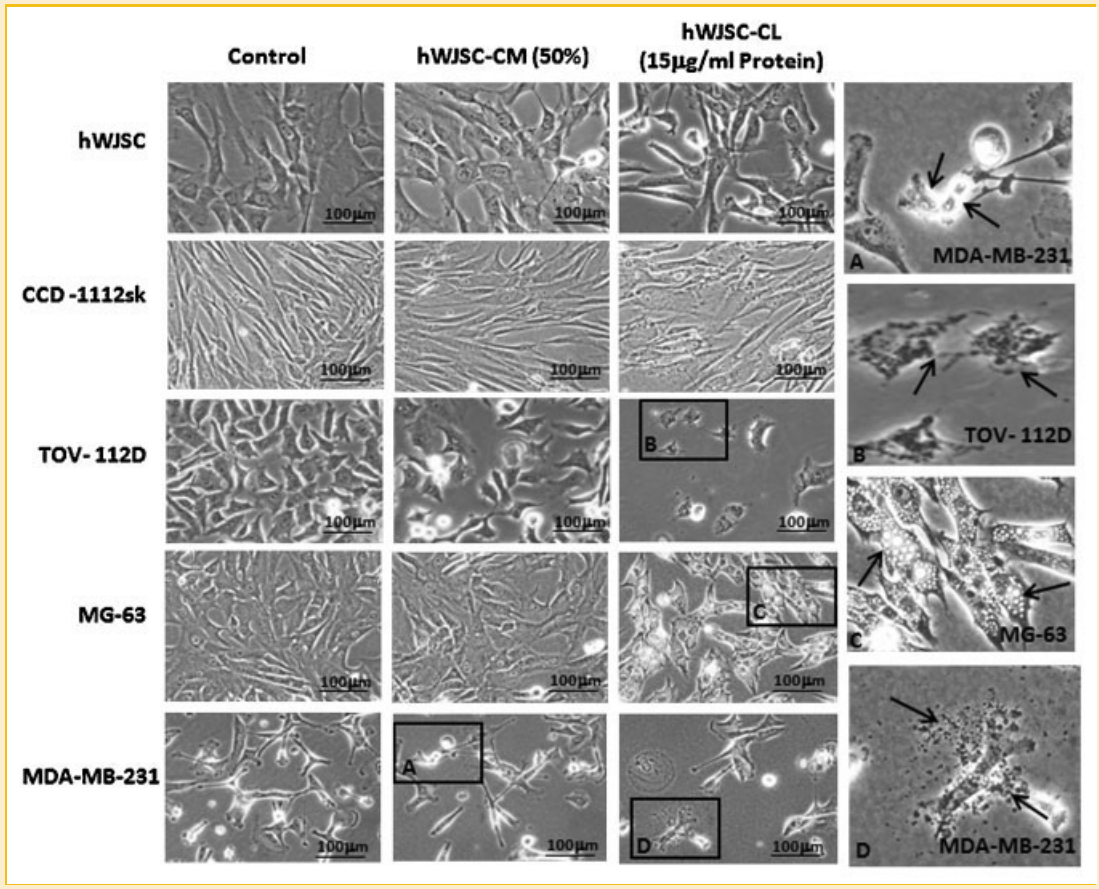


Fig. 1. Phase contrast images of control cell lines [human Wharton's Jelly stem cells (hWJSCs), human foreskin fibroblasts (CCD-1112 sk)] and human cancer cell lines [ovarian adenocarcinoma (TOV-112D), osteosarcoma (MG-63), and breast adenocarcinoma (MDA-MB-231)] cultured in hWJSC culture medium (control), 50% hWJSC conditioned medium (hWJSC-CM) and in hWJSC lysate containing 15 μg/ml protein (hWJSC-CL) for 72 h with fresh changes of hWJSC-CM and hWJSC-CL at 48 h. hWJSC and CCD-1112 sk continued to maintain their typical short and long fibroblastic morphology, respectively, and exhibited normal growth and proliferation in all three culture conditions. TOV-112D, MG-63, and MDA-MB-231 demonstrated varying morphological changes and decrease in cell numbers when cultured in hWJSC-CM and hWJSC-CL compared to controls. Boxed areas of the cancer cell lines are enlarged and arrows point at MDA-MB-231 cells showing shrinkage, degeneration, and varying degrees of membrane damage (A,D); TOV-112D cells showing fragmentation and numerous granular particles (B); and MG-63 cells showing increased vacuolation (C; scale bar 100 μm).

was inhibited by 36.93%, 39.97%, and 32.82% for 50%, 75%, and 100% hWJSC-CM, respectively. However, only the decreases in cell proliferation observed for MG63 in 75% hWJSC-CM and MDA-MB-231 in 50%, 75%, and 100% hWJSC-CM were statistically significant (Fig. 2A).

All three cancer cell lines cultured in hWJSC-CL (5, 10, or 15 μg/ml protein) showed dose-dependent inhibition of cell proliferation at 72 h. TOV-112D was inhibited by 36.84%, 56.84%, and 60.0%; MG-63 by 40.78%, 46.27%, and 60.27%, and MDA-231 by 39.66%, 46.36%, and 49.10% for the three concentrations of hWJSC-CL (5, 10, or 15 μg/ml), respectively, compared to their respective controls. These decreases in inhibition were statistically significant (Fig. 2B). Compared with the hWJSC group TOV-112D was decreased by 1.64%, 35.26%, and 38.42%; MG-63 was inhibited by 17.49%, 27.89%, and 40.76%, and MDA-MB-231 was inhibited by 25.81%, 36.47%, and 33.02% for 50%, 75%, and 100% hWJSC-CM, respectively. Except for the decrease observed for TOV-112D with hWJSC-CL at 5 μg/ml protein, all other decreases were statistically significant (Fig. 2B).

**BrdU assay.** The BrDU assay also showed that the TOV-112D, MG-63, and MDA-MB-231 cell lines cultured in hWJSC-CM (50%) and hWJSC-CL (15 μg/ml protein) produced inhibition of cell proliferation at 72 h compared to controls. TOV-112D was inhibited by 8.33% and 34.33%; MG-63 by 15.72% and 44.0%, and MDA-231 by 13.64% and 40.91% for hWJSC-CM (50%) and hWJSC-CL (15 μg/ml protein), respectively. However, only the decreases in inhibition observed following hWJSC-CL were statistically significant (Fig. 2C).

## CELL CYCLE ANALYSIS

The control cells (hWJSCs and CCD-1112 sk) showed their normal typical cell cycle profile when cultured in hWJSC-CM (50%) and hWJSC-CL (15 μg/ml protein) for 72 h. All three cancer cell lines exposed to hWJSC-CM (50%) showed increases in the sub-G1 phase of the cell cycle compared to the controls. The increases in sub-G1 phase for TOV-112D, MG-63, and MDA-MB-231 were 5.01%, 3.83%, and 9.01%, respectively, and only the increases observed for TOV-112D and MDA-MB-231 were statistically significant.

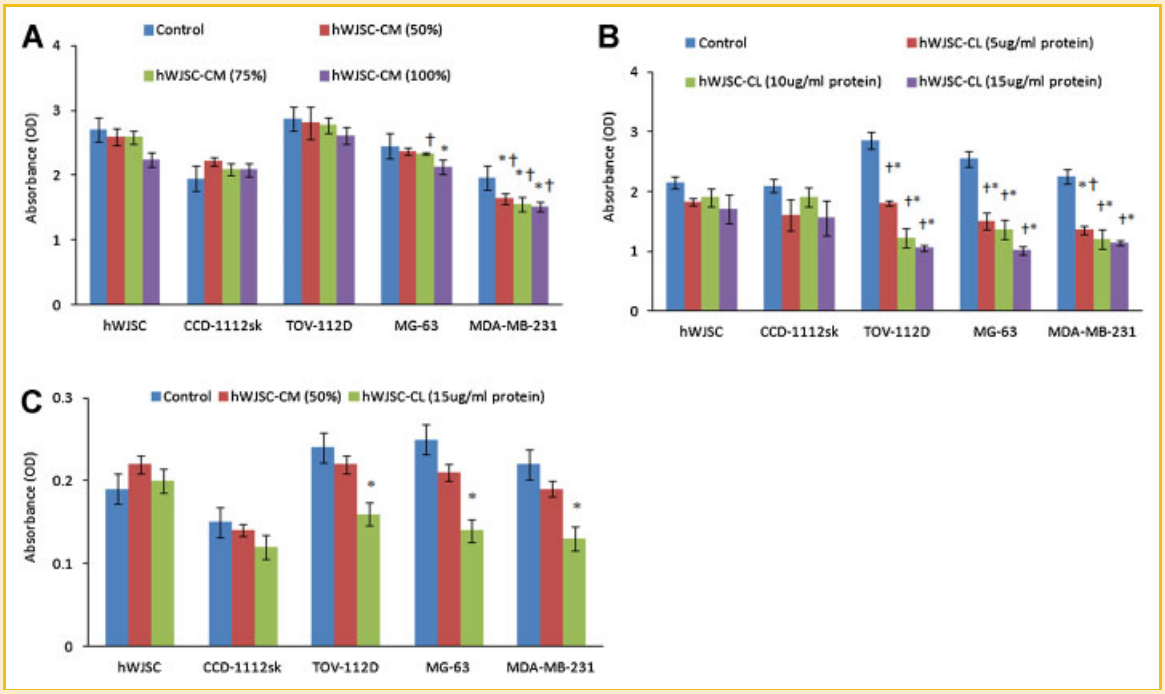


Fig. 2. A,B: Cell proliferation (MTT assay) of control cell lines [human Wharton's Jelly stem cells (hWJSCs) and human foreskin fibroblasts (CCD-1112 sk)] and human cancer cell lines [ovarian adenocarcinoma (TOV-112D), osteosarcoma (MG-63), and breast adenocarcinoma (MDA-MB-231)] cultured in hWJSC conditioned medium (50%, 75%, and 100% hWJSC-CM), in hWJSC lysate (hWJSC-CL containing 5, 10, or 15 μg/ml protein) for 72 h with fresh changes of the hWJSC-CM and hWJSC-CL at 48 h. C: Cell proliferation (BrdU assay) of the same cell lines as above cultured in hWJSC-CM (50%) and in hWJSC-CL (15 μg/ml protein) for 72 h with fresh changes of hWJSC-CM and hWJSC-CL at 48 h. All values were expressed as mean ± SEM from three different replicates. The "*" and "†" indicate statistical significance at $P < 0.05$ compared to their respective controls within the group and between the groups, respectively. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

Increases in the G2/M phase were seen only with MG-63 (3.92%) and MDA-MB-231 (4.58%; Fig. 3).

Similarly, all three cancer cell lines cultured in hWJSC-CL (15 μg/ml protein) showed increases in the sub-G1 phase by 9.41%, 9.73%, and 15.52% for TOV-112D, MG-63, and MDA-MB-231, respectively, compared to controls. Increases in G2/M phase were seen only with MG-63 (3.73%) and MDA-MB-231 (9.02%) and only the increases observed for MDA-MB-231 were statistically significant (Fig. 3).

## ANNEXIN V-FITC ASSAY

All three cancer cell lines (TOV-112D, MG-63, and MDA-MB-231) showed increases in annexin V-FITC positive cells when cultured in hWJSC-CM (50%) and hWJSC-CL (15 μg/ml protein) compared to the respective controls. The increases with hWJSC-CM (50%) were 4.06%, 5.65%, and 13.35% for TOV-112D, MG-63, and MDA-MB-231, respectively, and only the increases for MDA-MB-231 were statistically significant. Similarly, the increases with hWJSC-CL (15 μg/ml protein) were 14.18%, 12.08%, and 17.52% for TOV-112D, MG-63, and MDA-MB-231, respectively, and all these increases were statistically significant (Fig. 4).

## CELL MIGRATION ASSAY

In the first 24 h of culture, the cell migration assay showed that cell migration was evident in the cancer cell lines (TOV-112D, MG-63, and MDA-MB-231) and the control cell lines (hWJSC and CCD-1112 sk) on stimulation with 20% FBS subsequent to overnight starvation in serum-free medium. Cell migration of the cancer and control cell lines were inhibited by cytochalasin B which was indicative of impairment in the formation of microfilaments needed for motility. After 24 h of culture, the migration of TOV-112D, MG-63, and MDA-231 cells was inhibited by hWJSC-CM (50%) and hWJSC-CL (15 μg/ml protein) compared to controls and greater cell death and cell debris was observed with the cancer cells compared to controls (Fig. 5A). Lesser number of viable cells migrated into the lower chamber of the transwell following treatment with hWJSC-CM (50%) and hWJSC-CL (15 μg/ml protein) compared to controls as indicated by the viability assay. The mean decreases in viability of the migrated cells were 26.08%, 21.35%, and 21.14% for hWJSC-CM and 38.93%, 46.56%, and 32. 97% for hWJSC-CL, respectively, compared to their controls and these decreases in cell viability were statistically significant (Fig. 5B).

## TUNEL ASSAY

The control cell lines (CCD-1112 sk and hWJSCs) cultured in hWJSC-CM (50%) and hWJSC-CL (15 μg/ml protein) for 48 h were negative for TUNEL staining. TOV-112D and MDA-231 cells treated with hWJSC-CM (50%) and hWJSC-CL (15 μg/ml protein) for 48 h showed TUNEL positive cells, while the osteosarcoma cells (MG-63) were TUNEL negative (Fig. 6A).

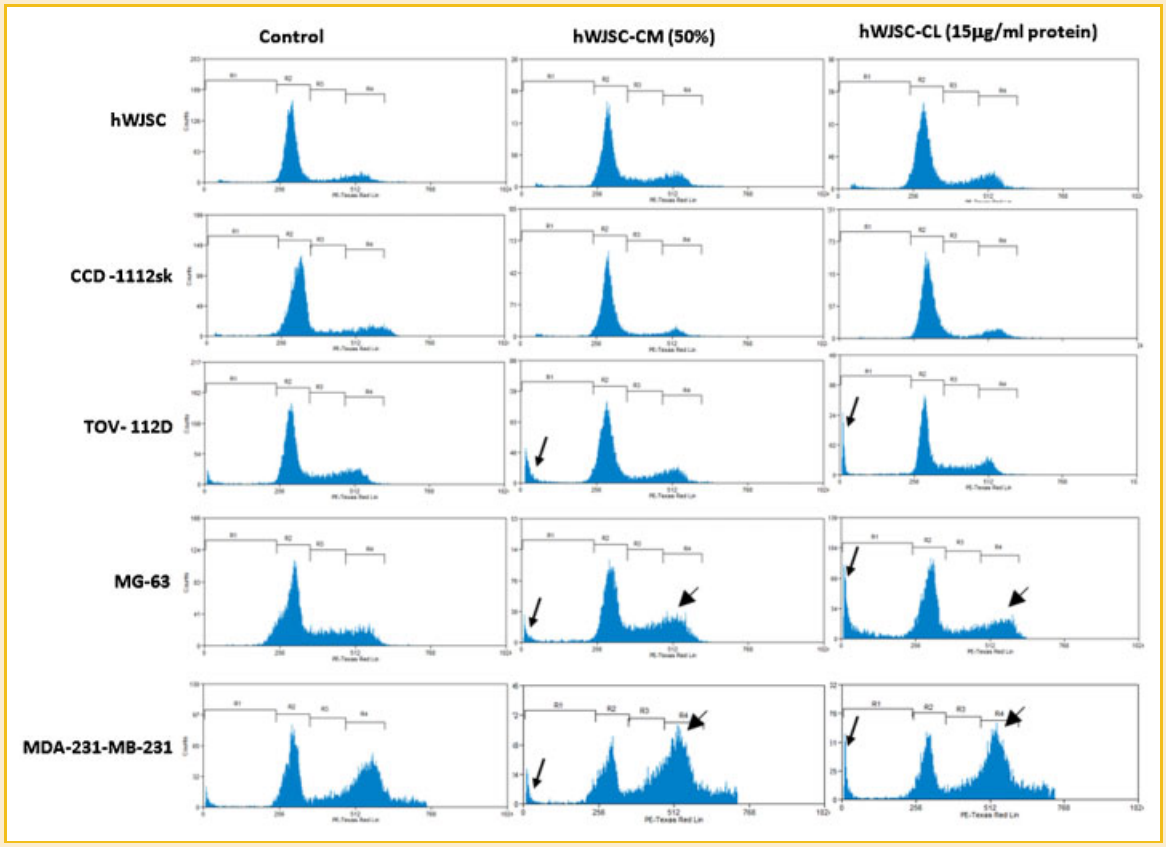


Fig. 3. Representative cell cycle flow cytometry images of control cell lines [human Wharton's Jelly stem cells (hWJSCs) and human foreskin fibroblasts (CCD-1112 sk)] and human cancer cell lines [ovarian adenocarcinoma (TOV-112D), osteosarcoma (MG-63), and breast adenocarcinoma (MDA-MB-231)] cultured in hWJSC culture medium (control), 50% hWJSC conditioned medium (hWJSC-CM) and in hWJSC lysate containing 15 μg/ml protein (hWJSC-CL) for 72 h with fresh changes of the hWJSC-CM and hWJSC-CL at 48 h. Thick arrow-heads represent increased peaks in M phase indicative of active mitosis and thin arrow-heads represent increased peaks in the sub-G1 phase indicative of apoptosis in the cancer cell lines compared to the controls. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

### IMMUNOHISTOCHEMISTRY

Exposure of MG-63 cells to hWJSC-CM (50%) for 72 h showed minimal cell inhibition and cell death (Fig. 6B:a,b) and weak positive staining for the autophagy-related proteins (anti-BECLIN-1 and anti-LC3B; Fig. 6B:ai,bi). However, when exposed to hWJSC-CL (15 μg/ml protein), the MG-63 cells became highly vacuolated, were markedly inhibited (Fig. 6B:c,d) and showed strong positive staining for anti-Beclin-1 and anti-LC3B (Fig. 6B:ci,di).

### QUANTITATIVE REAL-TIME POLYMERASE CHAIN REACTION (qRT-PCR)

Treatment of TOV-112D, MG-63, and MDA-MB-231 with hWJSC-CM (50%) and hWJSC-CL (15 μg/ml protein) for 72 h showed increased expression of the pro-apoptotic BAX gene and decreased expression of the anti-apoptotic BCL2 and SURVIVIN genes. Treatment of the cancer cells with hWJSC-CM (50%) demonstrated increases in BAX by 1.6-, 1.7-, and 2.4-fold for TOV-112D, MG-63, and MDA-MB-231, respectively. The anti-apoptotic gene BCL2 was decreased by 9.5- and 9.8-fold for MG-63 and MDA-MB-231, respectively, and SURVIVIN was decreased by 2.7-, 8.3-, and 9.4-fold for TOV-112D, MG-63, and MDA-231, respectively (Fig. 7A). Exposure to hWJSC-CL (15 μg/ml protein) showed increases in pro-apoptotic BAX gene expression of 1.8- and 2.4-fold for TOV-112D and MDA-MB-231, respectively, while BCL2 expression was decreased by 6.9-, 9.7-, and 9.5-fold and SURVIVIN decreased by 7.5-, 8.6-, and 9.7-fold for TOV-112D, MG-63, and MDA-MB-231, respectively (Fig. 7B).

The expression of the autophagy-related genes ATG5, ATG7, and BECLIN-1 were studied in the MG-63 cancer cell line. The autophagy-related genes ATG5 and BECLIN-1 had increased expression by 2.4- and 2.2-fold in MG-63 cells following treatment with hWJSC-CM (50%; Fig. 7C). Exposure of MG-63 cells to hWJSC-CL (15 μg/ml protein) showed increased expression of the autophagy-related gene ATG7 by 1.2-fold and decreased expression of ATG5 and BECLIN-1 by 9.0- and 1.1-fold, respectively (Fig. 7D).

## DISCUSSION

There is ample evidence that BMMSCs and other MSC types abolish tumor growth in vitro and in vivo. The injection of BMMSCs in a mouse model of Kaposi's sarcoma inhibited tumor growth in a dose-dependent fashion [Khakoo et al., 2006]. More recently, human

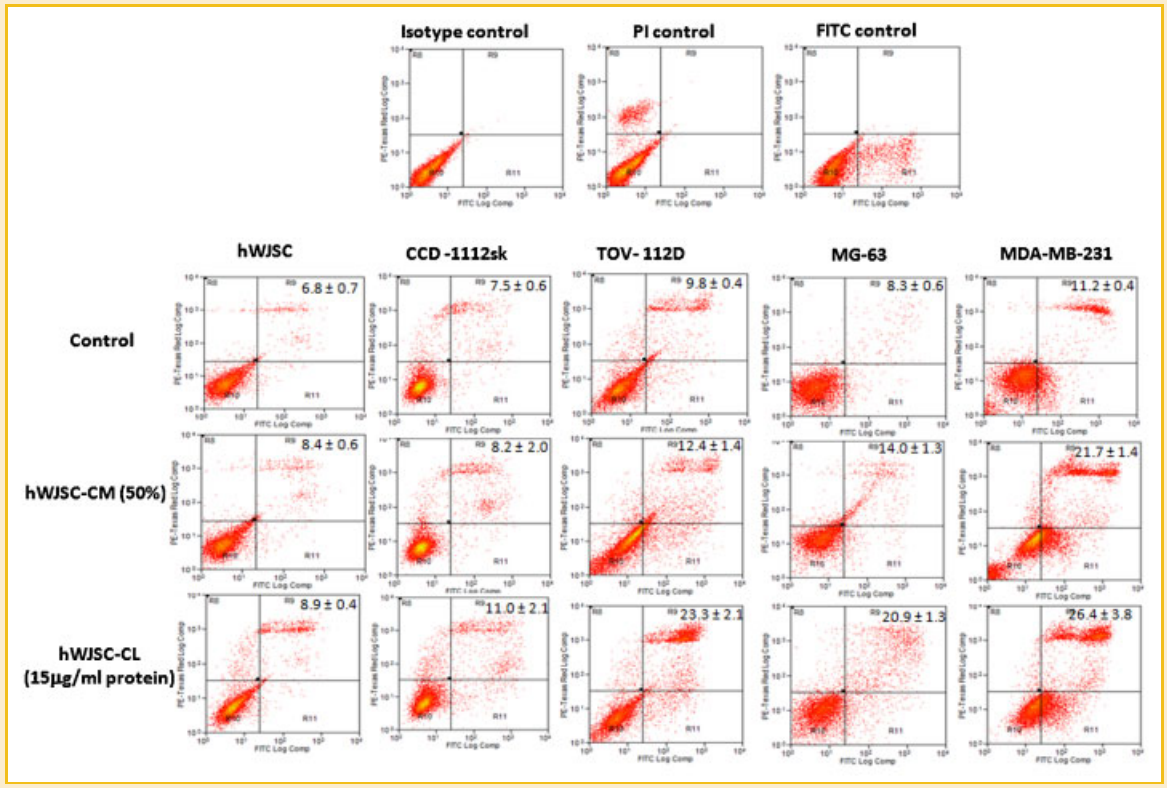


Fig. 4. Representative contour plots (annexinV-FITC flow cytometry) of control human cell lines [human Wharton's Jelly stem cells (hWJSCs) and human foreskin fibroblasts (CCD-1112 sk)] and human cancer cell lines [ovarian adenocarcinoma (TOV-112D), osteosarcoma (MG-63), and breast adenocarcinoma (MDA-MB-231)] cultured in hWJSC culture medium (control), 50% hWJSC conditioned medium (hWJSC-CM), and in hWJSC lysate containing 15 µg/ml protein (hWJSC-CL) for 72 h with fresh changes of the hWJSC-CM and hWJSC-CL at 48 h. AnnexinV-FITC positive cells were expressed as mean ± SEM from three different replicates. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

BMMSCs (hBMMSCs) displayed anticancer activity in SCID mice bearing disseminated non-Hodgkin's lymphoma xenografts [Secchiero et al., 2010]. Anti-tumor effects were also demonstrated in MSCs harvested from the UCMSCs. Ganta et al. [2009] showed that when rUCMSCs were injected intra-tumorally into rat breast carcinomas induced in the rats, the tumors were abolished within 6 weeks and later Ayuzawa et al. [2009] confirmed that hUCMSCs also completely abolished breast carcinomas in immunodeficient mice when injected intravenously or intra-tumorally.

We isolated and fully characterized a homogeneous population of MSCs specifically from the Wharton's jelly of the human umbilical cord (hWJSCs) [Fong et al., 2007, 2010a]. In pilot studies, we observed that when our hWJSCs were microinjected into green fluorescent protein-tagged liver tumors (GFP-HepG2) generated within embryoid bodies [Fong et al., 2009], the GFP-HepG2 cells were attenuated with time when monitored by confocal microscopy. In follow-up pilot studies, we also observed that when equal numbers of our hWJSCs were cocultured with GFP-HepG2 cells, the GFP-HepG2 cells were inhibited in culture. Cell coculture studies are sometimes unreliable because of possible artefactual growth advantages of one cell type over the other induced by the culture environment rather than a genuine anticancer effect. To exclude this possibility we focused in the present study on the role of the hWJSC extracts (hWJSC-CM and hWJSC-CL) rather than the cells per se. Fibroblast CM and CL were not used as controls because previous studies confirmed that such fibroblast CM and CL failed to affect cancer cell growth [Mukaida et al., 1991; Heylen et al., 1998].

The results of previous studies using cell coculture and that of the present study on cell extracts confirm that hWJSCs have anticancer effects mediated via cell-to-cell and non-cellular contact. It has been reported that the regulatory elements in cell extracts are known to influence various signaling mechanisms, such as initiation of transcriptional activity, differential expression of functional genes, and re-programming of specific cell types [Håkelien and Collas, 2002; Taranger et al., 2005]. However, the putative molecules in the extracts responsible for altering the cell fate remains largely unknown. The results of the present study showed that the CL had greater tumor inhibitory effects than the cell CM. This could be attributed to the presence of numerous regulatory molecules present within the cell compared to the limited number of molecules secreted into the medium because of varying sizes and cellular trafficking. The anticancer effects of hWJSC-CL and hWJSC-CM were superimposed in some cases probably because of the sensitivity and variations between assays and the differences cell numbers, cell doubling times, and incubation periods between the assays.

Our results are also consistent with the findings of Ganta et al. [2009] and Ayuzawa et al. [2009] where the same breast carcinoma cell line used in their studies was also inhibited by hWJSCs in our studies. However, we show that the anticancer effects of hWJSCs are

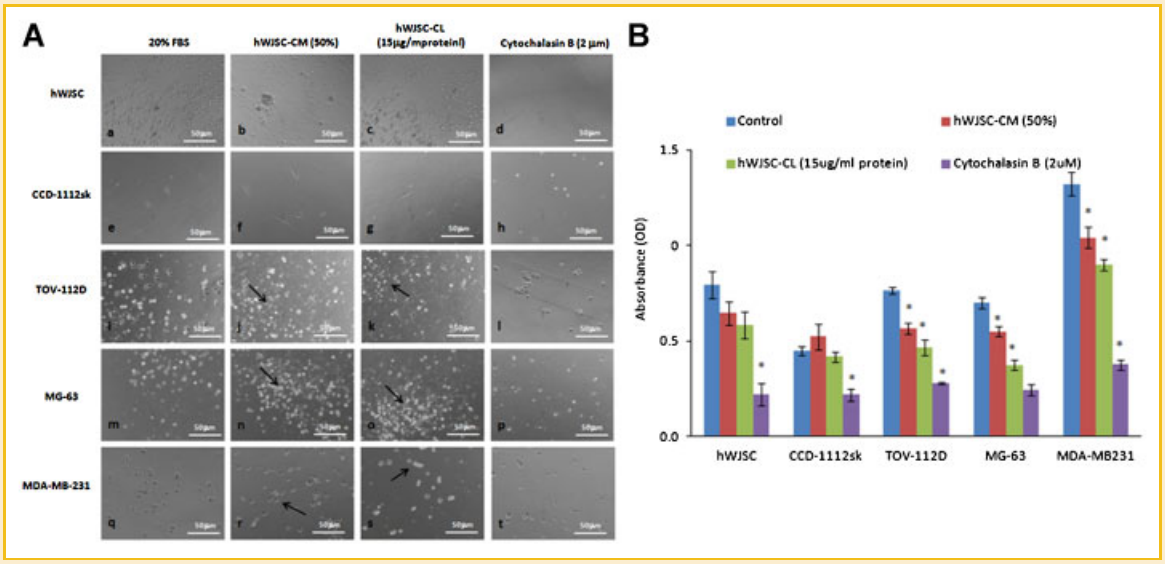


Fig. 5. Cell migration (Migration assay) of control human cells lines [human Wharton's Jelly stem cells (hWJSCs) and human foreskin fibroblasts (CCD-1112 sk)] and human cancer cell lines [ovarian adenocarcinoma (TOV-112D), osteosarcoma (MG-63), and breast adenocarcinoma (MDA-MB-231)] when initially cultured for 24 h in serum-free medium and subsequently exposed to hWJSC medium containing 20% FBS or 50% hWJSC-CM or hWJSC-CL-15 μg/ml protein. Cytochalasin B (2 μM) was used as a positive control. A: Representative phase contrast images showing the migrated cells in the lower Transwell chamber. TOV-112D, MG-63, and MDA-MB-231 demonstrated greater cell migration after stimulation with 20% FBS (i, m, q). Lesser cell migration and greater cell death/debris (indicated by arrows) were observed in TOV-112D, MG-63, and MDA-231 exposed to hWJSC-CM and hWJSC-CL compared to their respective controls (j, k, n, o, r, s; scale bar 50 μm). B: The migrated cell numbers are indicated as absorbance following MTT assay. All values were expressed as mean ± SEM from three different replicates and asterisks (*) indicate statistical significance at $P < 0.05$ compared to their respective controls, respectively. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

not limited to only breast carcinoma cells but also to other cancer cell types, such as ovarian carcinoma (TOV-112D) and osteosarcoma (MG-63). It was also interesting to note that the degree of anticancer activity varied between the cancer cell lines with the effects on osteosarcoma being most severe and ovarian carcinoma least. This could be attributed to the susceptibility of each cancer cell type to single or multiple mechanisms of inhibitory action because the phenotypic changes observed between the cancer types varied from cell shrinkage, fragmentation, blebbing, vacuolation, membrane damage, and the acquisition of numerous granular particles.

The observation that TOV-112D, MG-63, and MDA-MB-231 had increased G2/M cell populations with more cells in the mitotic phase compared to controls are similar to the study by Ayuzawa et al. [2009], who also showed increases in G2/M cell populations when MDA-MB-231 cells were co-cultured with UCMSCs. The cell cycle is basically governed by two regulatory systems, viz., cyclin-dependent kinase-mediated phosphorylation/dephosphorylation and checkpoint regulation via cell cycle regulators, such as P53, P21, or P27 [Collins et al., 1997; Rao et al., 1999]. It is possible that the hWJSCs disrupt these cell cycle regulatory mechanisms in the cancer cells.

The increased peaks in the sub-G1 phase and positive annexin V-FITC and TUNEL staining were suggestive that the hWJSC anticancer effect was via an apoptotic mechanism for some cancer cell types (TOV-112D and MDA-231). Interestingly, MSCs engineered to express TNF-related apoptosis inducing ligand (TRAIL) were found to inhibit cancer cells and together with traditional chemotherapeutic agents they induced apoptosis of squamous and lung cancer cell lines [Loebinger et al., 2010] and brain gliomas in mice [Cavarretta et al., 2010]. The increased expression of the pro-apoptotic BAX gene with decreased expression of anti-apotoic BCL2 and SURVIVIN in TOV-112D, MG-63, and MDA-231 cells were also indicative of an increased activity of apoptotic signaling pathways. SURVIVIN is highly expressed in cancers [Olie et al., 2000] and its inhibition was claimed to cause spontaneous apoptosis of lung cancer cells [Sato et al., 2004].

The mechanism of the anticancer effect on the osteosarcoma cell line (MG-63) was different and more unique with accumulation of an increased number of vacuolations reminiscent of an autophagic process. Autophagic cell death is dependent on the ATG genes with ATG-7 and BECLIN-1 being the key genes that are involved in autophagic cell death in mammalian cells [Yu et al., 2004]. TGF-β was reported to activate autophagy by upregulation of ATG5, ATG7, and BECLIN-1 [Kiyono et al., 2009]. TGF-β was also involved in the accumulation of autophagosomes and conversion of microtubule associated protein 1 light chain 3 (LC3) leading to autophagy [Kiyono et al., 2009]. The positive expression of MG-63 cells for BECLIN-1 and LC3B therefore further confirms that the effect of hWJSCs on MG-63 cells was via autophagy-related cell death. However, in the present study, increased ATG5 and BECLIN-1 gene expression was seen only with hWJSC-CM and not with hWJSC-CL. Perhaps, autophagy related genes are expressed in the earlier phase of cell death and final cell death is mediated via an apoptotic pathway as reflected by increased sub-G1 phase of cell cycle and increased Annexin V-FITC positive cells following treatment with hWJSC-CL.

The unique and intense anticancer effect of hWJSCs on MG-63 may also be related to perturbations of hyauronic acid (HA) and

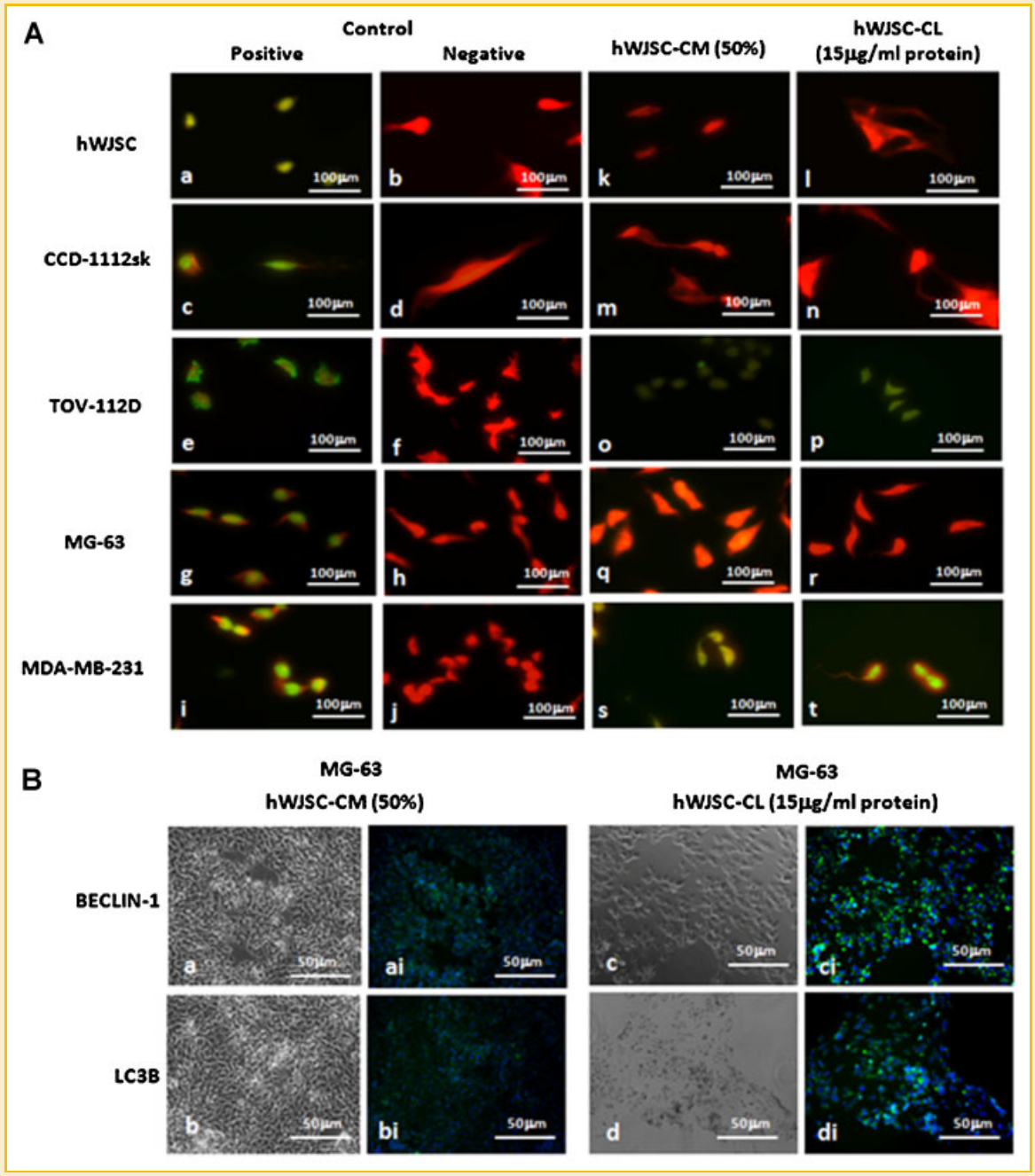


Fig. 6. A: Terminal deoxynucleotidyl transferase biotin-dUTP nick end labeling (TUNEL) images of control human cell lines [human Wharton's Jelly stem cells (hWJSCs) and human foreskin fibroblasts (CCD-1112 sk)] and human cancer cell lines [ovarian adenocarcinoma (TOV-112D), osteosarcoma (MG-63), and breast adenocarcinoma (MDA-MB-231)] cultured in hWJSC culture medium (control), 50% hWJSC conditioned medium (hWJSC-CM), and in hWJSC lysate containing 15 μg/ml protein (hWJSC-CL) for 48 h. Positive TUNEL staining was observed with TOV-112D and MDA-MB-231 for hWJSC-CM and hWJSC-CL (o, p, s, t). DNAse treated cells were used as positive controls (scale bar 100 μm). B: Immunohistochemistry images of human osteosarcoma cells (MG-63) treated with BECLIN-1 and LC3B primary antibodies following culture in 50% hWJSC-CM and in hWJSC-CL (15 μg/ml protein) for 72 h with fresh changes of the hWJSC-CM and hWJSC-CL at 48 h. Positive staining was observed for BECLIN-1 (ai, ci) and LC3B (bi, di) following culture in hWJSC-CM and hWJSC-CL. Alexa Fluor 588 was the secondary antibody and the cells were counterstained with DAPI (4′-6-diamidino-2-phenylindole; scale bar 50 μm). [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

glycosoaminoglycans (GAGs) homeostasis because hWJSCs are known to naturally secrete large amounts of these two agents [Fong et al., 2011a]. Hyaluronan oligosaccharides were shown to inhibit tumorigenicity of two osteosarcoma cell lines (MG-63 and LM-8) [Hosono et al., 2007] and chondroitin sulphate and dermatan sulphate inhibited cell proliferation of osteoblasts and osteosarcoma cells [Nikitovic et al., 2005].

Cytokines such as TNF-α, TGF-β, and interferon-γ are known to influence cell cycle regulation in cancer cells and induce growth attenuation and apoptosis. IL-16 an immunomodulatory cytokine

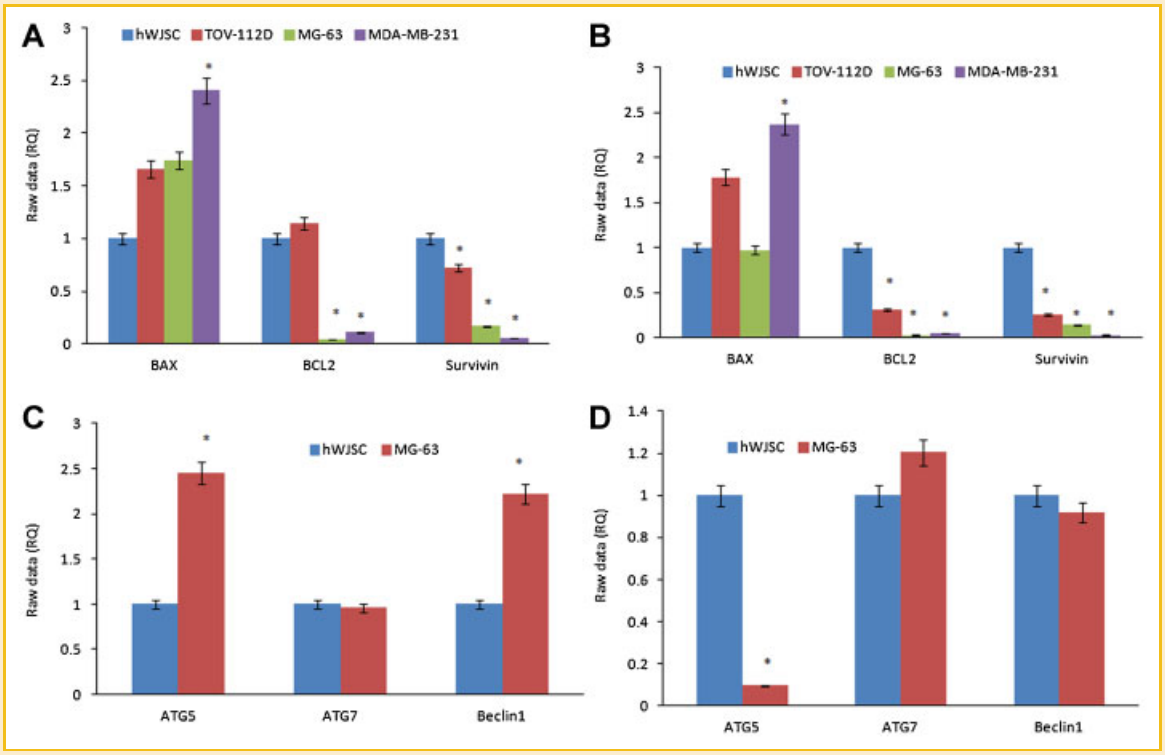


Fig. 7. Gene expression profiles (qRT-PCR) of the apoptotic-related genes (BAX, BCL2, and SURVIVIN) of human cancer cell lines [ovarian adenocarcinoma (TOV-112D), osteosarcoma (MG-63), and breast adenocarcinoma (MDA-MB-231)] cultured in 50% hWJSC-CM (A) and in hWJSC-CL containing 15 μg/ml protein (B) for 72 h. Gene expression profiles of the autophagy-related genes (ATG5, ATG7, and BECLIN-1) in the human osteosarcoma cell line (MG-63) following culture in 50% hWJSC-CM (C) and in hWJSC-CL containing 15 μg/ml protein (D) for 72 h. Data analysis and relative quantitation was done using the comparative $C_t$ ($\Delta\Delta C_t$) method. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

was shown to inhibit TNF-α, IFN-γ, and IL-1β in the murine model of rheumatoid arthritis [Klimiuk et al., 1999] and hWJSCs were shown to have increased expression of IL-16 in xenotransplantation studies [Gauthaman et al., 2011]. Our group also showed recently that hWJSC-CM contains very high concentrations of members of the interleukin family (IL-1a, IL-6, IL-7, and IL-8) and the adhesion molecule ICAM-1 [Fong et al., 2011b]. The receptors of many of such adhesion molecules (ICAM-1) have been observed on MSCs as well as leucocytes for their roles of adhesion to endothelial cells and transmigration. MSCs in general can migrate across endothelium and function like leukocyte trafficking [Yen and Yen, 2008]. It is therefore possible that hWJSC-CM and hWJSC-CL exert their influence on cell cycle regulation leading to cell cycle arrest probably through cytokine and adhesion molecule-mediated mechanisms.

In transwell coculture and soft agar colony assays where Lewis lung cancer cells (LLC) were not in direct contact with rUCMSCs, LLC growth was inhibited implying involvement of a diffusible factor/s secreted by the UCMSCs. This factor was shown to be associated with the regulation of the cell cycle [Maurya et al., 2010]. MSCs engineered to express modified IL-12 were shown to induce T-cell responses and anti-tumor effects in melanomas in mice [Seo et al., 2011]. Thus, various mechanisms and signaling pathways may be involved in the interplay between different cancer cell types and hWJSCs giving different degrees of anticancer effects. In a detailed comparative microarray transcriptome profiling of our hWJSCs we reported that genes related to immune modulation (interferon family), tumor suppression and pro-apoptosis (interleukin family) including IL-12A [Fong et al., 2011b] were highly expressed and therefore IL-12A may be one of the molecules involved in the apoptotic-mediated cell death seen with some cancers in this study. Interestingly, interferon-β (IFB-β) is also known to strongly inhibit tumor cell growth and induce apoptosis in vivo [Wong et al., 1989; Chawla-Sarkar et al., 2001]. Furthermore, recently, Matsuzuka et al. [2010] showed that hUCMSCs expressing IFN-β significantly attenuated bronchiolalveolar carcinoma xenografts in SCID mice and culture medium conditioned with IFN-β–hUCMSCs significantly attenuated cell growth of H358 and SW1573 cell lines in vitro. Addition of anti-human IFN-β monoclonal antibody to the CM neutralized the attenuating effect. hWJSCs were shown to have anti-inflammatory properties with reduced expression of inflammatory mediators TNF-α and IFN-γ [Moodley et al., 2009].

The anticancer effects of hWJSCs and their extracts bring a new perspective to cancer cell biology and therapeutics that merits further studies. Studies are in progress to confirm the in vitro results on in vivo animal models. Interestingly, it is well known that hBMMSCs transform into tumor-associated fibroblasts (TAFs) when in the neighborhood of breast cancer tumors and are involved in enhancement and metastasis of the tumor. However recently, we showed that even though hWJSCs may have a common origin to

hBMMSCs during early embryonic development, they did not participate in TAF formation and may therefore not be linked to enhanced growth and metastasis of solid tumors [Subramanian et al., 2012]. Thus the non-tumorigenicity [Gauthaman et al., 2011] and anti-tumorigenicity properties of hWJSCs coupled with its inability to transform to the TAF phenotype makes it an attractive and safe stem cell for use in future clinical application.

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