

Primary ovarian cancer cell inhibition by human Wharton's Jelly stem cells (hWJSCs): Mapping probable mechanisms and targets using systems oncology

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Abstract:

Ovarian cancer is one of the most lethal gynaecological cancers. Its subtle onset and absence of symptoms in early stages are associated with poor prognosis and high mortality. Identification of early biomarkers would aid in ovarian cancer control. Mesenchymal stem cells (MSCs) and/or their secretory products are identified to have cancer inhibitory properties. Therefore, it is of interest to study the anticancer properties of human Wharton's jelly stem cells conditioned medium (hWJSCs-CM) on primary ovarian carcinoma cells *in vitro*. Primary cultures of epithelial ovarian carcinoma cells (EOCs) and hWJSCs were used in this study. EOCs were exposed to hWJSC-CM (100%) for 24h-72h and changes in morphology and cell proliferation were monitored. Treatment with hWJSC-CM showed altered morphological changes that resulted in death of EOCs. Colorimetric assay [MTT, (3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide)] showed mean decreases in EOC proliferation by 16.21%, 23.89% and 40.08% at 24h, 48h and 72h respectively compared to control. Ingenuity Pathway Analysis (IPA, Ingenuity Systems, USA) deduced important molecular pathways and signaling networks associated with cancer cell death and these correlated with significant expression of tumour suppressors and apoptotic genes in hWJSCs. Secretory products of hWJSC-CM induced cell death of EOCs *via* apoptosis. IPA identification of canonical genes/pathways involved in EOCs that overlap with hWJSCs tumour suppressors and apoptosis genes further support this hypothesis. Additional *in vitro* and *in vivo* studies are necessary to validate EOCs inhibition with hWJSC-extracts towards their mechanism of action.

Keywords: Ovarian cancer, hWJSCs, Cell proliferation, Cell death, IPA

Background:

Cancer is a leading cause of death world-wide. The 2012 cancer statistics estimated about 1638,910 new cancer cases and 577,190 cancer related deaths in the United States [1]. Among the cancers in women, ovarian cancer is the most lethal gynaecological cancer and is associated with high mortality [2]. Ovarian cancers arise from the covering surface epithelium of the ovary. The epithelial ovarian cancer (EOC) is classified into five types based on their histopathology and includes high grade serous carcinomas; clear cell carcinomas; endometrioid

carcinomas; clear cell carcinomas and low-grade serous carcinomas [3]. Its subtle onset and absence of symptoms in early stages are associated with poor prognosis and high mortality. Treatment is effective with better prognosis at early diagnosis. In contrast, failure rates with chemotherapy are high with advanced EOCs and also the mortality.

Many factors are involved in the pathogenesis of EOCs. Given the cellular heterogeneity and signaling complexities in EOCs, use of standard chemotherapeutic regimen may not be always successful. This is reflected in the 5 year survival rate which

depicts a plateau in the last 15 years [4]. New class of anticancer agents such as monoclonal antibodies, small molecules and protein kinase inhibitors that have different mechanisms of action and targets have either been instituted in clinical practice or currently undergoing clinical trials [5, 6]. Unlike

conventional anticancer agents that target mostly the DNA breaks, synthesis or mitosis, these newer agents target the cellular signaling pathways.

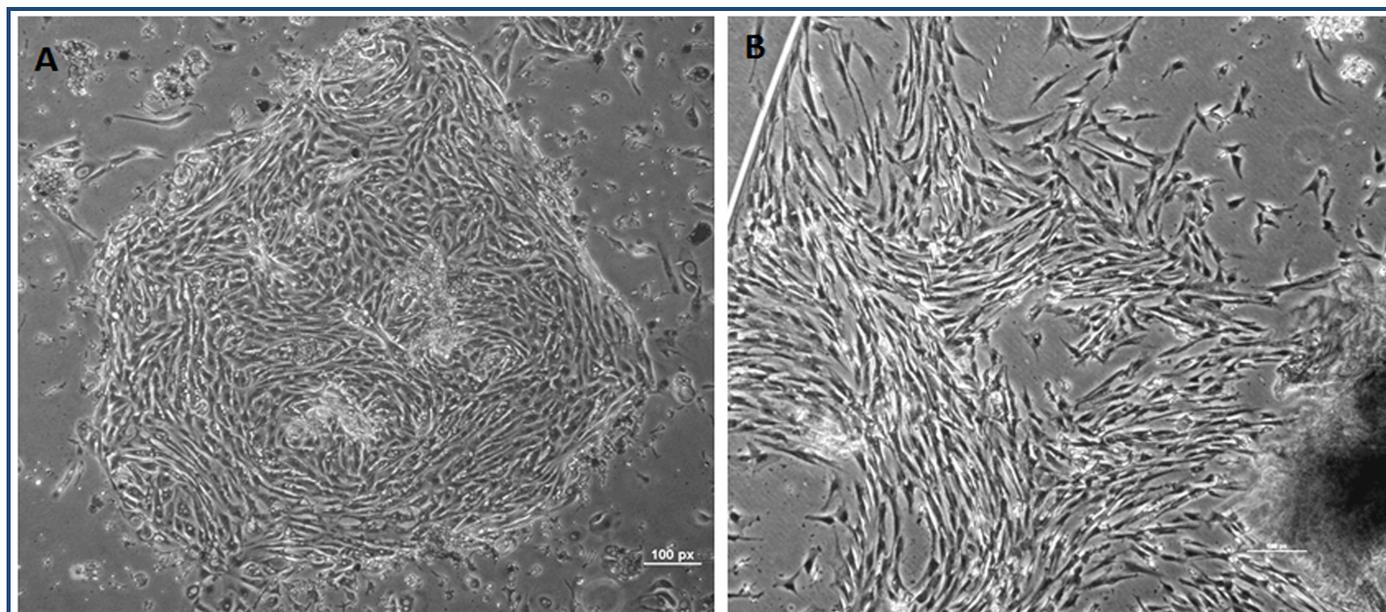


Figure 1: Phase contrast microscopic images showing primary cultures of **A)** human epithelial ovarian cancer cells (EOCs) and **B)** human Wharton's Jelly stem cells (hWJSCs). EOCs demonstrated epithelioid morphology and cobble stone appearance, while the hWJSCs showed short fibroblastic morphology. Magnification 10X.

Interestingly, mesenchymal stem cells (MSCs) which holds great potential in regenerative medicine has been reported to have anticancer properties in both *in vitro* and *in vivo* experimental systems [7-11]. Since MSCs have homing abilities, they have been used as vehicles to deliver genes or small molecules that have anticancer effects to the tumor sites to help tumour suppression [12]. Intravenous administration of bone marrow MSCs, led to successful homing and abrogation of Kaposi's sarcoma in mice [9]. Unengineered MSCs derived from the umbilical cord matrix have demonstrated inhibition of mammary breast adenocarcinoma *in vitro* and *in vivo* [11].

The underlying mechanisms of most cancer inhibitions by these stem cells are not fully understood. It remains to be known whether stem cells per se or their secretomes bring about the inhibitory effects of cancer cells. As such in the present study the effects of human Wharton's jelly stem cells conditioned medium (hWJSC-CM) on primary epithelial ovarian carcinoma cells were evaluated with regard to their changes in morphology and cell proliferation.

Methodology:

Approval for use of human ovarian cancer tissue and umbilical cords were obtained from the King Abdulaziz University (KAU) Ethical Committee [33-15/KAU].

Derivation of Epithelial ovarian cancer cells (EOCs)

Human ovarian cancer tissues were obtained following informed consent from patients undergoing surgery at the Department of Obstetrics and Gynaecology, King Abdulaziz University Hospital (KAUH), Jeddah, Saudi Arabia. The samples were collected and transferred to the laboratory in a sterile flask containing Hanks Balanced Salt Solution

(Invitrogen Life Technologies, Carlsbad, CA) and 1% antibiotics/antimycotic. The samples were processed within 3 h - 6 h. Primary cultures of epithelial ovarian cancer cells (EOCs) were established according to earlier reported protocol [13] with slight modifications. Briefly, the samples were washed twice with PBS to remove the blood and tissue debris. The ovarian surface side was placed down on prewarmed Dispase (Sigma, ST Louis, Missouri, USA) and incubated for 30 minutes at 37°C and gently shaken every 10 min. Dispase activity was stopped using RPMI 1640 containing 10% fetal bovine serum (FBS). The contents were transferred to 15 ml conical Falcon tube and centrifuged at 500 g x 5 min. The supernatant was carefully removed and the resuspended cells plated in 100 mm petri dishes (Becton Dickinson, Franklin Lakes, NJ) and cultured using RPMI 1640 media supplemented with 10% FBS, 2mM Glutamax, 1% antibiotics [penicillin (50 IU/ml), streptomycin (50 µg/ml)] at standard culture conditions of 37°C in a 5% CO₂ incubator. The cultures were left undisturbed until cell growth was evident, with media changes every 72 h.

Derivation of human Wharton's Jelly stem cells (hWJSCs)

Human umbilical cords were obtained following informed consent from patients undergoing full term delivery at the Department of Obstetrics and Gynecology, KAUH. The umbilical cord was transferred in a sterile container containing HBSS and antibiotics and processed within 6 h- 12 h. Briefly, the umbilical cord was cut into pieces of ~2 cm and opened length wise. The blood vessels were removed and the opened side exposed to an enzyme cocktail containing collagenase Type-I (2 mg/mL), collagenase Type-IV (2 mg/mL) and Hyaluronidase (100 IU) for 30 minutes. The enzyme activity was blocked by addition of medium containing 10% FBS, and the matrix contents were gently scraped onto the medium. The

medium containing cells and matrix substance was centrifuged at 500 g x 5 min. The supernatant was discarded and the contents were washed twice with PBS. The resultant pellet was finally resuspended in culture media comprised of DMEM high glucose, supplemented with 10% FBS, 2 mM Glutamax, 1% non-essential aminoacids, basic fibroblast growth factor 16

ng/mL and 1% antibiotics [penicillin (50 IU/ml), streptomycin (50 µg/ml)] and incubated at standard culture conditions of 37°C in a 5% CO₂ incubator. The cultures were left undisturbed until cell growth was evident, with media changes every 72 h.

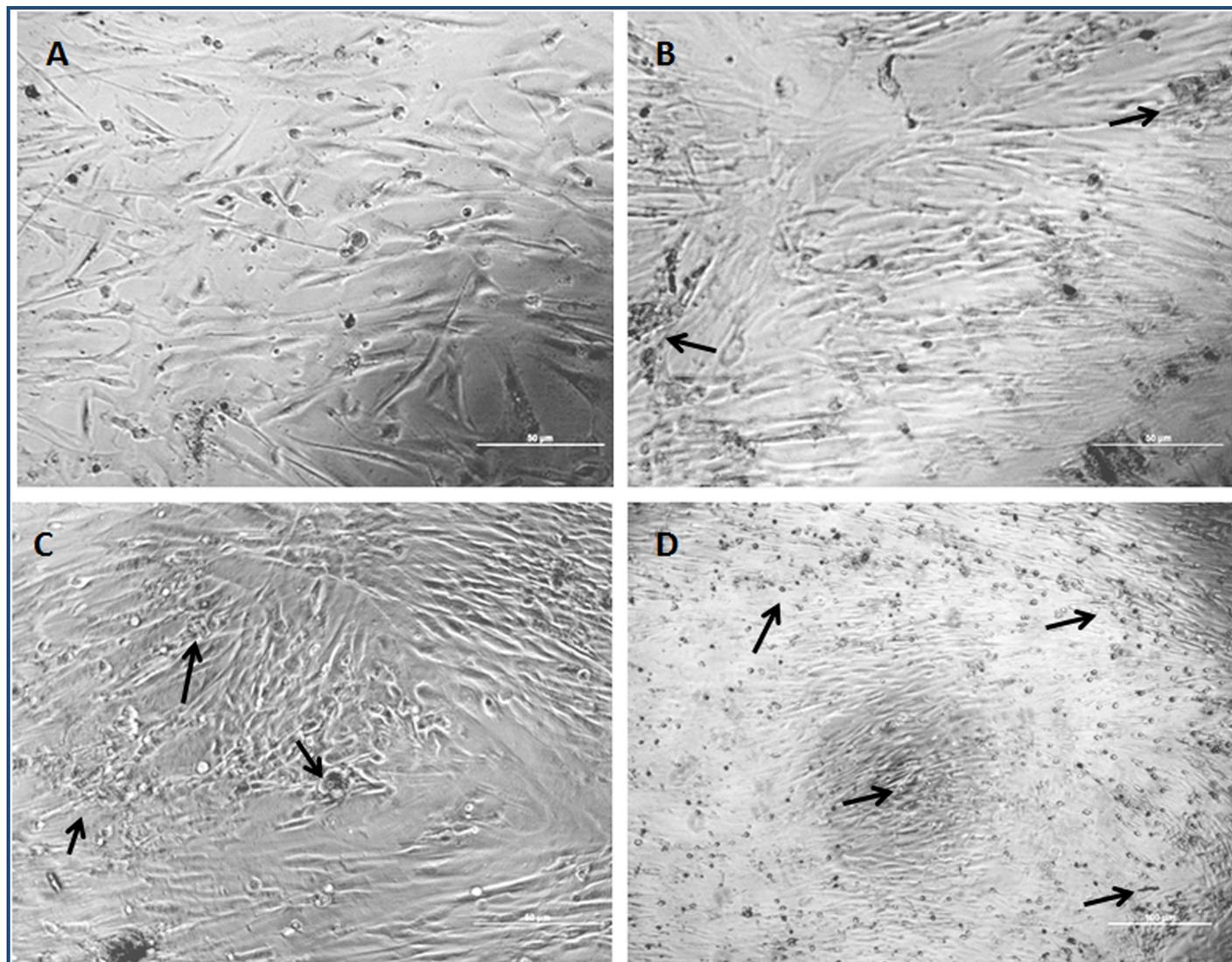


Figure 2: Phase contrast microscopic images of primary cultures of human epithelial ovarian cancer cells (EOCs) following treatment with human Wharton's Jelly stem cells conditioned medium (hWJSC-CM, 100%) at 24h (B); 48h (C) and 72h (D) compared to untreated control (A). Decrease in cell numbers and changes in morphology leading to cell death (indicated by arrows) were observed in the treated groups. Magnification 10X.

hWJSC-Conditioned Media

Derived hWJSCs were grown under standard culture conditions and the media changed every 48 h. When the cells were 70% confluent the culture media was removed and fresh media was added and the cells cultured for upto 72 h. The hWJSC-conditioned media (hWJSC-CM, 100%) was then harvested, filter sterilized using 0.2 µm syringe filters and stored in 4°C as aliquots until further use in experiments.

Cell morphology of epithelial ovarian cancer cells (EOCs) cultured in hWJSC-CM

EOCs were plated at a seeding density of 2x10⁴ cells/well of a 24 well plate and allowed to attach overnight. The media was removed and hWJSC-CM (100%) was added to the

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experimental wells, while the controls were maintained in standard culture media. Both controls and experimental groups were cultured at 37°C in a 5% CO₂ incubator for up to 72 h. Cell morphology were imaged every 24 h using phase contrast microscopy.

Proliferation of epithelial ovarian cancer cells (EOCs) cultured in hWJSC-CM

EOCs seeded and cultured as above were analyzed for their proliferation using MTT assay at 24 h, 48 h and 72 h according to manufacturer's instructions. Briefly, the culture media was removed and 200 µl of fresh media containing 10 µl of MTT reagent (3-(4, 5-dimethylthiazolyl)-2, 5-diphenyltetrazolium bromide; Sigma, MO) was added and incubated under

standard culture conditions for upto 4 h. The media was removed and 200 μ l of solubilization reagent was added. Absorbance was obtained at 570 nm with a reference wavelength of 630 nm using a SpectraMaxi3 Multimode Reader (Molecular Devices, USA).

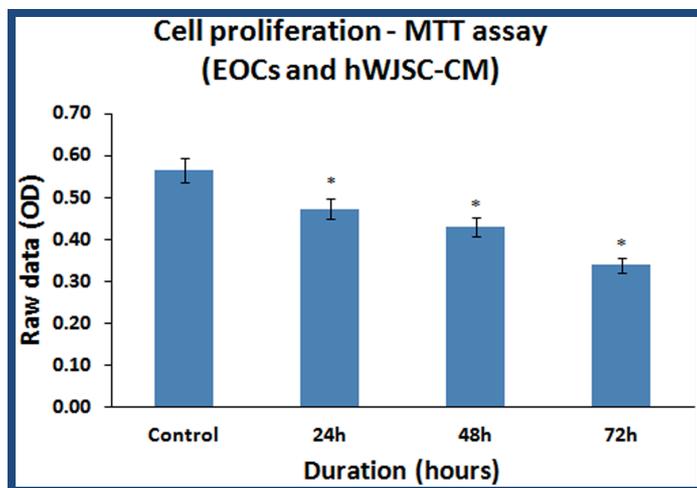


Figure 3: Cell proliferation (MTT assay) of primary cultures of human epithelial ovarian cancer cells (EOCs) following treatment with human Wharton's Jelly stem cells conditioned medium (hWJSC-CM, 100%) for 24h, 48h and 72h. Mean decreases in cell proliferation were observed and these decreases were statistically significant compared to the control. The values are expressed as mean \pm SEM of three different experiments. * indicates statistical significance ($p < 0.05$).

Ingenuity pathway analysis

Functional core analysis to predict networks that are involved in EOCs were performed using Ingenuity Pathway analysis (IPA) software, (Ingenuity Systems, USA). Details of the genes involved in EOC [Janus kinase 2 (JAK2), Kirsten rat sarcoma viral oncogene homolog (KRAS), v-myc avian myelocytomatosis viral oncogene homolog (MYC), phosphatidylinositol-4,5-bisphosphate 3-kinase, catalytic subunit alpha (PIK3CA), B-cell CLL/lymphoma 2 (BCL2), tumor protein p53 (TP53) and phosphatase and tensin homolog (PTEN), cyclin-dependent kinase inhibitor 2A (CDKN2A), ATM serine/threonine kinase (ATM)] were imported into the IPA software to predict signaling mechanisms, targets and their association to genes expressed by hWJSCs. Network predictions based on gene input were generated using algorithms contained in Ingenuity Knowledge Base. Fischer's Exact test was done to calculate the p-value indicating the probability of each biological function associated with the network.

Results:

Primary cultures of EOCs and hWJSCs

Primary cultures of EOCs were established from the ovarian surface epithelium. The cells demonstrated their characteristic epithelial cobble stone morphology (Figure 1A) in initial passages and they had irregular shapes in later passages. Isolates from primary ovarian cancer tissue expanded in culture yielded several million cells upon reaching confluence usually by 5-7 days. Primary cultures of hWJSCs demonstrated their characteristic short fibroblastic morphology in culture (Figure 1B). The hWJSCs also demonstrated good proliferation and positive expression of MSC related CD markers (Data not shown).

Morphological changes of EOCs exposed to hWJSC-CM

The EOCs exposed to hWJSC-CM (100%) demonstrated varying changes in morphology including decrease in size and fragmentation of cells leading to cell death (Figure 2A-D). The cells appeared thin and elongated and had lost their characteristic epitheloid shape in cultures. There was also a decrease in cell numbers in the treated group compared to controls at 24 h, 48 h and 72 h (Figure 2A-D).

Proliferation EOCs exposed to hWJSC-CM

EOCs exposed to hWJSC-CM (100%) showed time dependent decrease in cell proliferation at 24 h, 48 h and 72 h compared to the untreated controls (Figure 3). The mean decreases observed in EOCs were 16.21%, 23.89% and 40.08% for 24 h, 48 h and 72 h respectively compared to the untreated control. These decreases in cell proliferation were statistically significant ($p < 0.05$) compared to the control.

Ingenuity pathways analysis of EOC genes

IPA of EOC molecules (735) using both direct and indirect relationships from Ingenuity Knowledge Base reference sets identified top canonical pathways, upstream regulators, diseases, biological and toxicological functions following core analysis of the molecules. The ovarian cancer signalling involves 133 genes of which 35 (26.3%) are implicated in EOC and the epithelial mesenchymal transition pathways comprise of 184 genes of which 35 (19.0%) are involved. The top upstream regulators were EGF, P53, Beta-estradiol, TGF- α 1 and SP1. Screening for top diseases and biological functions revealed 735 molecules associated with cancer and organismal injury, 253 molecules in cellular movement and about 350 molecules in both cell death/survival. Important overlapping canonical pathways of ovarian cancer genes with other diseases is depicted in Figure 4A.

Discussion:

EOC is most lethal gynaecological malignancy and depending on the cancer subtype diverse signalling pathways are activated. To better understand the tumour development and progression, a two pathway model, where the cancer is classified as either Type I and Type II is used. Type I involves low grade serous, mucinous, endometrioid and clear cell tumors. They evolve from benign cystadenomas or borderline lesions and later become malignant. Type II tumours involve high grade serous, high grade endometrioid and undifferentiated tumours that progress rapidly and also is associated with peritoneal involvement [13]. Mutations that play significant role in the pathogenesis of ovarian carcinoma include BRCA1, BRCA2, MLH1, K-Ras, PIK3CA, PTEN and P53 [14, 15]. Mutations in BRCA1 and BRCA2 are involved with more than 90% of the hereditary ovarian cancers. Activation of K-Ras mutation is associated with angiogenesis via ERK/MAPK pathway. Mutations of PTEN or PIK3CA may lead to malignant transformation and uncontrolled growth via activation of PTEN/PI3K/mTOR pathway [14, 15].

In general, ovarian cancer treatment is aimed at debulking surgery followed by use of chemotherapy with carboplatin and paclitaxel. In addition it was recently outlined that addition of anti-VEGF monoclonal antibody bevacizumab was found to improve progression-free survival [16, 17]. Two clinical trials which included patients with FIGO stage III-IV ovarian cancer and had macroscopic residual disease following initial surgery (GOG-218 trial) or in patients who had poor prognosis, and

those with optimally or sub-optimally debulked tumour (ICON7 trial) showed improved progression-free survival in patients who received bevacizumab [16, 17]. A recent study identified that addition of bevacizumab did not increase overall survival in whole study population, but some benefits were observed in patients who had poor prognosis [18]. Ovarian cancers are mostly diagnosed at a late stage, as in early stages

the symptoms are obscure making their diagnosis difficult. As such most currently available therapies although help reduce disease symptoms the mortality rate still remains high. Early diagnosis has good prognosis and therefore identification of early biomarkers in ovarian cancer or novel molecules that might help treatment are essential.

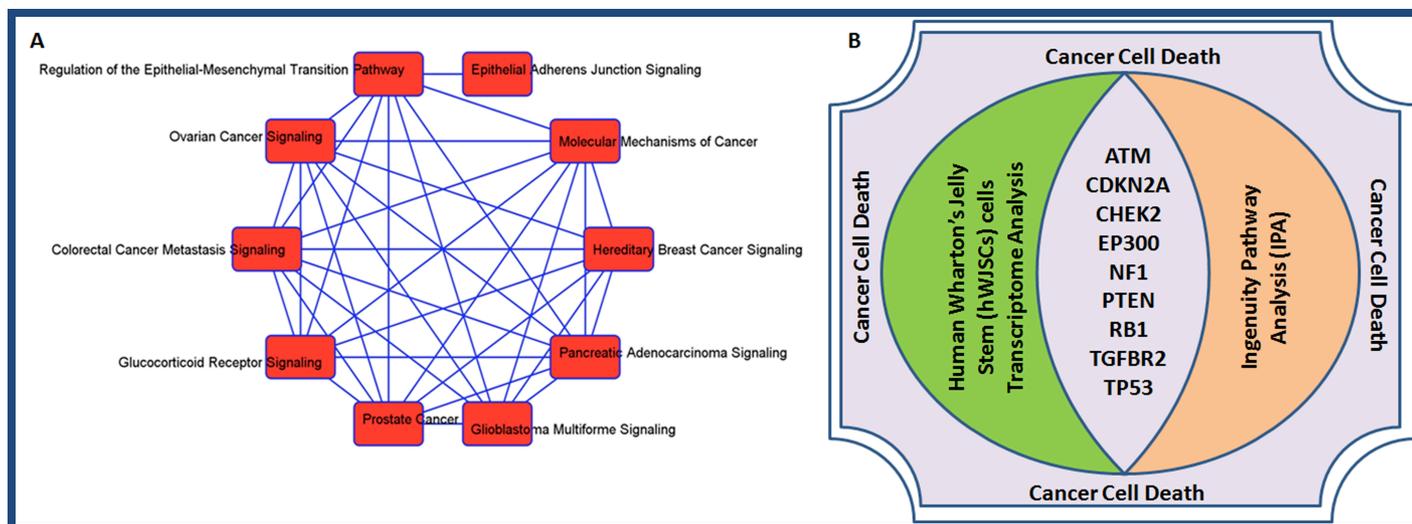


Figure 4: A - Ingenuity pathway analysis of genes involved in human epithelial ovarian cancer (EOCs) and other associated diseases that have overlapping pathways. B - Some of the common overlapping genes/molecules that are expressed by hWJSCs that are related to cell death mechanisms.

Some recent studies have identified that umbilical cord derived MSCs including the hWJSCs have inhibitory effects on various cancers either *in vitro* or *in vivo* [8, 19, 20]. In the present study too we identified that hWJSC-CM inhibit the primary epithelial ovarian cancer cells (EOCs, **Figure 3**). As there were no direct contact of the stem cells (hWJSCs) with EOCs, any inhibitory action observed is probably due to the factors secreted by hWJSCs into the medium. Earlier transcriptome studies on hWJSCs have identified increased expression of tumour suppressor genes and apoptosis inducing genes [21]. There were significant overlap in the canonical pathways and molecular mechanisms in EOCs as identified by IPA with that of the transcriptome profile of hWJSCs indicating that hWJSCs might be targeting these pathways to bring about cancer cell inhibition (**Figure 4B**). Further proteomics studies of hWJSC-CM is however necessary to help identification of the putative molecule(s) that bring about cancer cell death.

Conclusions:

Results show that hWJSC-CM induce inhibition of primary EOCs *in vitro* and cause cell death. The IPA predictive results indicating the genes/targets involved in EOCs that overlap with hWJSCs tumour suppressors further support this hypothesis. Additional *in vitro* and *in vivo* studies are necessary to validate EOCs inhibition with hWJSC-extracts to identify putative molecules/targets of cancer inhibition.

Conflict of Interest:

All authors have no conflict of interests.

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