Establishment and Characterization of Primary Human Ovarian Cancer Stem Cell Line ($CD44^{+ve}$)

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Abstract: Ovarian cancer is ranked as the 7th most lethal cancer worldwide with 239,000 new cases annually. The mortality rate is high because most ovarian tumors are diagnosed at advanced stages and are resistant to chemotherapy and thus incurable due to the lack of effective early detection of ovarian tumors. There is a small sub-population of ovarian tumor cells capable of self-renewal and differentiation into different cancer cell types, called cancer stem cells (CSCs), which might be responsible for cancer relapse. The $CD44^{+}$ phenotype in ovarian tumor cells elucidates cancer initiating cell-like properties of promoting differentiation, metastasis, and chemotherapy-resistance. Increased expression of genes previously associated with CSCs promotes regenerative capacity by promoting stem cell function that can drive cancer relapse and metastasis. In this study we present a method to isolate the primary epithelial ovarian cancer cells from human solid tumor and establish $CD44^{+ve}$ primary ovarian cancer stem cell ($OCSC^{CD44+ve}$) line using magnetic microbeads. Also we evaluated the expression of stemness genes *Nanog*, *Sox2*, *Oct4*, and *Nestin* by real-time qPCR analysis. The quantitative analysis by real-time qPCR shows that $OCSC^{CD44+ve}$ overexpressed the embryonic stem cell marker genes *Nanog*, *Oct4*, *Sox2*, and *Nestin* when compared with ovarian cancer cells $OCC^{CD44-ve}$ as positive control and ovarian cells as negative control. We demonstrate that CD44 in malignant ovarian tumors is a critical molecule that exhibits cancer stem cell properties that enhance tumorigenicity and cancer metastasis. Our results provide a better understanding of ovarian CSCs, which is important for future *in vivo* studies with subsequent target therapy for preclinical studies.

Keywords: Ovarian cancer, Cancer stem cell, Stemness genes, CD44, Chemo-resistance.

INTRODUCTION

Ovarian cancer is considered as one of the most lethal gynecological cancers among women all over the world and is ranked as the 7th most lethal cancer worldwide with 239,000 new cases annually [1]. The fatality rate is high because ovarian cancer is usually diagnosed at late stage. This is mainly due to the cancer cells' resistance to current chemotherapeutic drugs and also is caused by the lack of prognostic means for early detection of ovarian tumors [2]. The cause of about 90% of cancer starting from the ovary epithelium is that stem cells reside in this area [3].

Carcinogenic tumor contains heterogeneous populations of cells: tumorigenic and non-tumorigenic. Tumorigenic cells can develop into tumors with high efficiency compared to non-tumorigenic cells from the same tumor specimen. Because they have similarities to normal adult stem cell properties like self-renewal and differentiation, these cancer-initiating cells are known as cancer stem cells (CSCs) [4].

CSCs initiating from DNA alterations in stem cells most likely undergo abnormal growth. Cancer stem-like cells may start from an environment with dominant growth enhancing signals instead of growth inhibiting signals [5]. CSCs also arise from normal stem cells or from normal progenitor cells. If CSCs are originated from normal stem cells, then the cancer cells exhibit the functional self-renewal pathway that is active in stem cells. But if the CSCs are originated from progenitor cells, then oncogenic alteration should be mixed reactivation of self-renewal pathway in the cancer cells [6].

CD44 is expressed in different types of cancer cells and cancer-initiating cells. There is a clear link between CD44 and cancer relapse and mortality [7]. CD44 is a surface transmembrane glycoprotein, is a hyaluronic acid receptor and a multi-structural and multi-functional cell surface molecule implicated in adhesive cell-cell and cell-matrix interactions. CD44 ultimately affects cancer proliferation, motility, and differentiation [8]. The *CD44* gene is located at chromosome 11 on the short arm (p13); 20 exons with length of 60 kb are implicated in the genomic organization of this molecule. The first 5 and the last 5 exons are stable and the middle 10 exons are subjected to alternative splicing [9]. The $CD44^{+}$ phenotype in ovarian tumor cells demonstrates CSC properties of promoting invasion, differentiation, and chemotherapy resistance. This $CD44^{+}$ phenotype

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is associated with the risk of cancer recurrence and metastasis in ovarian cancer patients [10].

Normal stem cell self-renewal is mainly regulated by essential transcription factor based pathways. During carcinogenesis, abnormal regulation of transcription factor expression can enhance abnormal growth, self-renewal, and differentiation of cancer cells. A subpopulation of tumor cells have these stem cell-like properties, and these cancer stem-like cells drive tumor progression. CSCs' phenotype is defined by special transcription factors such as Nanog, Sox2, Oct4, and Nestin [11].

The Nanog protein is encoded by the *Nanog* gene, which contains 4 exons and encompasses 8265 bp of DNA in humans. It is presented on the short arm of chromosome 12 (12p13.31). *Nanog* mRNA encode for a protein with a length of 305 amino acids with a MW of 34.6 kDa [12]. Nanog is a transcription factor that is well confirmed as a main regulator of self-renewal of embryonic stem cell. Nanog is implicated in enhancing carcinogenesis in part through regulation of the CSC subpopulation. High expression of Nanog is correlated with poorer prognosis in many ovarian malignancies. Nanog is elevated in CSCs and inhibition of Nanog drives the reduction of the CSC [13].

Sox2 (sex determining region Y-box2) protein is encoded by *Sox2* gene that is a single exon gene encompassing 2513 bp of DNA in humans and is presented on the long arm of chromosome 3 (3q26.3-q27). *Sox2* mRNA encode for a protein with a length of 317 amino acids with a molecular weight of 34.3 kDa [14]. Sox2 has been described as a transcription factor that is important for enhancing self-renewal of embryonic stem cells. Sox2 researchers have only recently turned their focus from embryogenesis to its role in cancer because it is absent in normal epidermis but begins to be expressed in most human cancers. Furthermore, the function of Sox2 in tumor progression has become important in oncology. Sox2 in various tumor progression and CSC function has been documented [15].

The Oct4 (octamer-binding transcription factor 4) protein is encoded by the *POU5F1* gene, which contains 6 exons and encompasses 16395 bp of DNA in humans and is presented on the short arm of chromosome 6 (6p21.31). *POU5F1* mRNA encodes for a protein with a length of 360 amino acids with a molecular weight of 38.6 kDa [16]. Oct4 is expressed by CSCs in various types of cancer and is associated with increased tumorigenic capacity. Oct4 has an important role in the initiation and progression of cancer and is associated with advanced tumor stage. Knockdown of expression of Oct4 in CSCs by RNA interference resulted in a significant inhibition in cell proliferation [17].

Nestin protein is encoded by the *Nes* gene. It contains 4 exons and encompasses 8635 bp of DNA in humans and is presented on the long arm of chromosome 1 (1q23.1). *Nes* mRNA encode for a protein with a length of 1621 amino acids with a MW of 177.4 kDa [17]. Nestin is a protein that is highly expressed in a variety of stem and progenitor cells, and its expression is tightly associated with rapidly proliferating progenitor cells during development and repair processes. It has been reported that Nestin regulates cell growth and survival of CSCs [18]. Cancer cells with elevated expression of Nestin drive aggressive malignant behavior correlated with poor prognosis [19].

Here we present a method to isolate from human solid ovarian tumor the primary epithelial ovarian cancer cells (OCCs) and establish $OCC^{CD44\text{-}ve}$ versus ovarian cancer stem cells ($OCSC^{CD44+ve}$) by using magnetic microbeads. We also evaluated the expression of Nanog, Sox2, Oct4, and Nestin by real-time qPCR analysis. Our studies provide characterization of $OCSC^{CD44+ve}$ that express key stemness genes, which might provide diagnostic/ prognostic and therapeutic targets in ovarian cancer.

METHODS

Culture media including DMEM, fetal bovine serum (FBS), L-glutamine, penicillin/streptomycin, and 0.05% trypsin-EDTA were used in culturing the various ovarian epithelial cells, cancer stem cells, and cancer cells. Cell culture medium, Collagenase A, and Anti-CD44 Human Magnetic Microbeads were obtained from Miltenyi Biotec, USA. For complete medium (CM), DMEM was complimented by 10% FBS, and 1% penicillin/streptomycin and 1% L-glutamine. CM was stored at 4°C and warmed to 37°C prior to use. Collagenase A was diluted in phosphate buffer saline, stored at -20°C, and warmed to 37°C before using.

Tissue Collection

A tissue sample (~10 g) from epithelial ovarian cancer tumor was obtained from the Surgical department, operative room, Mansoura University,

Mansoura, Egypt, from one patient (56 years old), who signed a consent form. Tumor sample was isolated from areas microscopically detected as cancer. A healthy ovarian tissue sample was obtained from the Gynecology and Obstetrics department of Mansoura University Hospital, Egypt, under IRB approved protocol from one subject (48 years old), who signed a consent form. Collected samples were placed into sterile 50 mL Falcon tubes containing DMEM supplemented with 5% penicillin-streptomycin. Samples were transported to culture lab within a half hour. Similar areas of tissues were fixed by formalin and embedded with paraffin. Histopathology analysis of sections to detect the subtype, grade, and stage of ovarian tumor (serous carcinoma; IIIC) were performed by a pathologist at Mansoura University.

Established Primary Cell Lines

We established epithelial ovarian cell (OC) line, epithelial ovarian cancer cell (OCC) line (CD44-ve cells), and epithelial ovarian cancer stem cell (OCSC) line (CD44+ve cells) for parallel and comparative characterizations.

Establishment of Primary Ovarian Cells (OC)

Tissue samples (~10 g) were cut by scalpel into many smaller pieces that were placed in 60 mm Petri-dishes containing 10 mL of DMEM and further cut into tiny pieces with scissors. For mechanical disruption, the specimen slurries were collected into 50 mL Falcon tubes and mechanical effects applied by a vortex mixer for 10 minutes. This was followed by enzymatic digestion by collagenase-A, mixed well, then into a water bath for 60 minutes at 37°C (125 rpm), until the cell slurries could be passed through a 100 µm mesh with a syringe plunger and centrifuged at 1500 *x g* for 7 minutes. Then supernatants were aspirated and cell pellets were collected.

Establishment of Primary OCSCs ($OCSC^{CD44+ve}$) by MACS® Separation

For magnetic labeling, cell number was determined where 80 µL of PBS was applied per 10^7 total cells, 20 µL anti-CD44 magnetic microbeads were added and incubated for 15 minutes in a refrigerator (2-8°C), then centrifuged for 10 minutes and the supernatant was aspirated. For magnetic separation, a column was prepared by rinsing with 3 mL PBS and then applied on the magnetic field. The cell suspension was placed onto the column and the flow was collected, which contained unlabeled cells ($OCC^{CD44-ve}$). The column was removed from the magnetic field and placed in a suitable collection tube. Finally 2 mL of PBS was rinsed onto the column and immediately the magnetically labeled cells ($OCSC^{CD44+ve}$) were flushed out by pushing the plunger into the column. The method was in accordance with Weigmann *et al.* [20].

Cell Culture

The 3 different types of cell lines underwent culture by CM and were incubated in a 37°C, 5% CO_2 incubator. The medium was changed every day after the 1st plating and every 3 days for the next 3 weeks. As cells reached confluency, they were sub-cultured to allow more space for continued growth by Trypsin-EDTA solution. Cells for each passage were counted and tested for viability by trypan blue dye exclusion assay.

Flow-Cytometry Characterization

To characterize tumor cells with CD44 and CD24 markers, OCSCs were collected after several days in culture and washed with phosphate buffer saline before mixing with antibodies. OCSCs were mixed with monoclonal-antibodies against human CD44-fluorescein isothiocyanate (FITC) and CD24-phycoerythrin (PE) and incubated on ice in the dark for 15 minutes. Then cells were washed with PBS and suspended 2×10^6 in 1 mL PBS for fluorescence-activated cell sorting.

Wound Healing Assay

$OCSC^{CD44+ve}$ and $OCC^{CD44-ve}$ were cultured in CM for 24 hours in 6-well plates to 70-80% confluence and then the cell layer was scratched in a vertical line with a tip. Then they were fed with fresh serum-free medium. Images were taken at 0 and 24 hours after scratching to demonstrate the cell mobility and migration. The procedure was performed in triplicate.

RNA Extraction and Real-Time qPCR Analysis

RNA was extracted from primary freshly isolated OC, $OCC^{CD44-ve}$, and $OCSC^{CD44+ve}$ cells using the TRIzol Reagent and purified by GeneJET™ RNA Purification Kit (Fermentas, USA). One µg of RNA from each sample was applied for reverse transcription using the Maxima® First Strand cDNA Synthesis Kit (Fermentas). Real-time qPCR was done with the Maxima® SYBR Green qPCR Master Mix (Sigma). Reactions were done in a 20 µL volume with 20 pmol primers. The primers for the *Nanog*, *Oct4*, *Sox2* and

Table 1: Primers Sequence of Stemness Genes *Nanog*, *Oct4*, *Sox2* and *Nestine*

Gene	Name	Sequences	Application
Nanog	NANOG-OLF	CTCACACGGAGACTGTCTCTC (MT=66)	qPCR (size= 147n)
	NANOG-OLQ	CTTGACCGGGACCTTGTCTTC (MT= 66)	
Oct4	OCT4-OLF	CGGAGGAGTCCCAGGACATCA (MT= 68)	qPCR (size= 144n)
	OCT4-OLQ	GGCTGAATACCTTCCCAAATAGA (MT= 64)	
Sox2	SOX2-OLF	GCCGAGTGGAAACTTTTGTCGG (MT= 68)	qPCR (size= 154n)
	SOX2-OLQ	GCAGCGTGTACTTATCCTTCTTC (MT= 68)	
Nestin	NESTIN –OLF	CTGAAAAGTTCCAGCTGGCTGT (MT= 66)	qPCR (size= 151n)
	NESTIN-OLQ	CCAGGAGGGTCCTGTACGTG (MT= 66)	

Nestin genes are listed in Table **1** using GAPDH as a control. PCR was performed by an initial denaturation at 95ºC for 10 minutes, followed by 40 cycles for 15 seconds at 95ºC, 30 seconds at TM-5Cº, and 30 seconds at 72ºC. Specificity was detected by melting curve analysis. PCR products were electrophoresed on 2% agarose gels. The Ct values of samples were used in the PCR data analysis.

Statistical Analysis

Data are presented as the mean ± SD. Student's t-test was used to calculate the variation between mean values. Differences among the groups with $P<0.05$ was considered to be statistically significant.

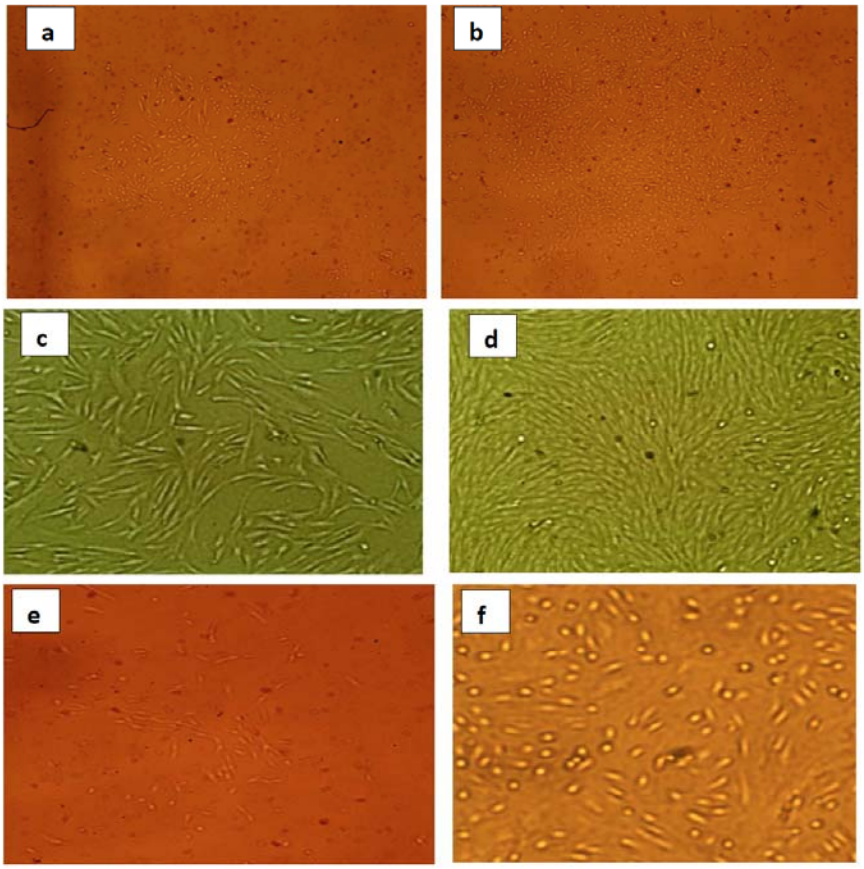

Figure 1: Morphologic characteristics of cultured cells.

(**a**) Representative clusters of $OCSC^{CD44+ve}$ after 3 days in culture; (**b**) Swirl-like clusters of $OCSC^{CD44+ve}$ cells spreading on the tissue culture plate after 5 days in culture, magnification 20X (inverted microscope); (**c**) $OCSC^{CD44+ve}$ cover the culture plate after 9 days in culture; (**d**) Confluent monolayer of $OCSC^{CD44+ve}$ after 12 days in culture with clear spindle shapes; (**e**) Representative $OCC^{CD44-ve}$ grown separately after 3 days in culture and used as positive control; (**f**) Representative semi-round shaped OCs after 12 days in culture were used as negative control, magnification 100 X (inverted microscope). *Ovarian Cancer Stem Cells (OCSC); Ovarian Cancer Cells (OCC); Ovarian Cells (OC)*

RESULTS

Morphological Characteristics of Primary OCSC$^{CD44+ve}$ and OCC$^{CD44-ve}$ Cultures

We detected the change in morphology of freshly-isolated OCSC$^{CD44+ve}$ derived from human solid tumor. Three days after culture in CM the OCSC$^{CD44+ve}$ started to adhere to the plate and this was indicated by the presence of small clusters (Figure **1a**). By day 5 (Figure **1b**), the clusters of OCSC$^{CD44+ve}$ formed swirl-like shapes and spread on the plate. By day 9, OCSC$^{CD44+ve}$ had clear slender, elongated, spindle shapes and covered the culture plate (Figure **1c**). By day 12, the OCSCs formed a confluent monolayer and it was sub-cultured to allow more space for continued growth (Figure **1d**). Three days after plating OCC$^{CD44-ve}$ in CM, the adherent OCC$^{CD44-ve}$ grew separately on the culture plate and were used as positive control (Figure **1e**). Healthy OCs freshly isolated from human ovary had a semi-rounded shape after 12 days in culture and were used as negative control (Figure **1f**).

Flow-Cytometry Characterization

We performed the flow cytometry experiment using antibodies against CD44 and CD24 cell surface markers. We obtained four subtypes of cancer cells (CD44-/CD24+, CD44+/CD24+, CD44-/CD24-, and CD44+/CD24-) as shown in Figure **2**. The percentages of CD44-/CD24+, CD44+/CD24+, CD44-/CD24-, and CD44+/CD24- cells were 0.3%, 14.4%, 6.4%, and 78.9%, respectively; thus 93.3% of cells expressed CD44+.

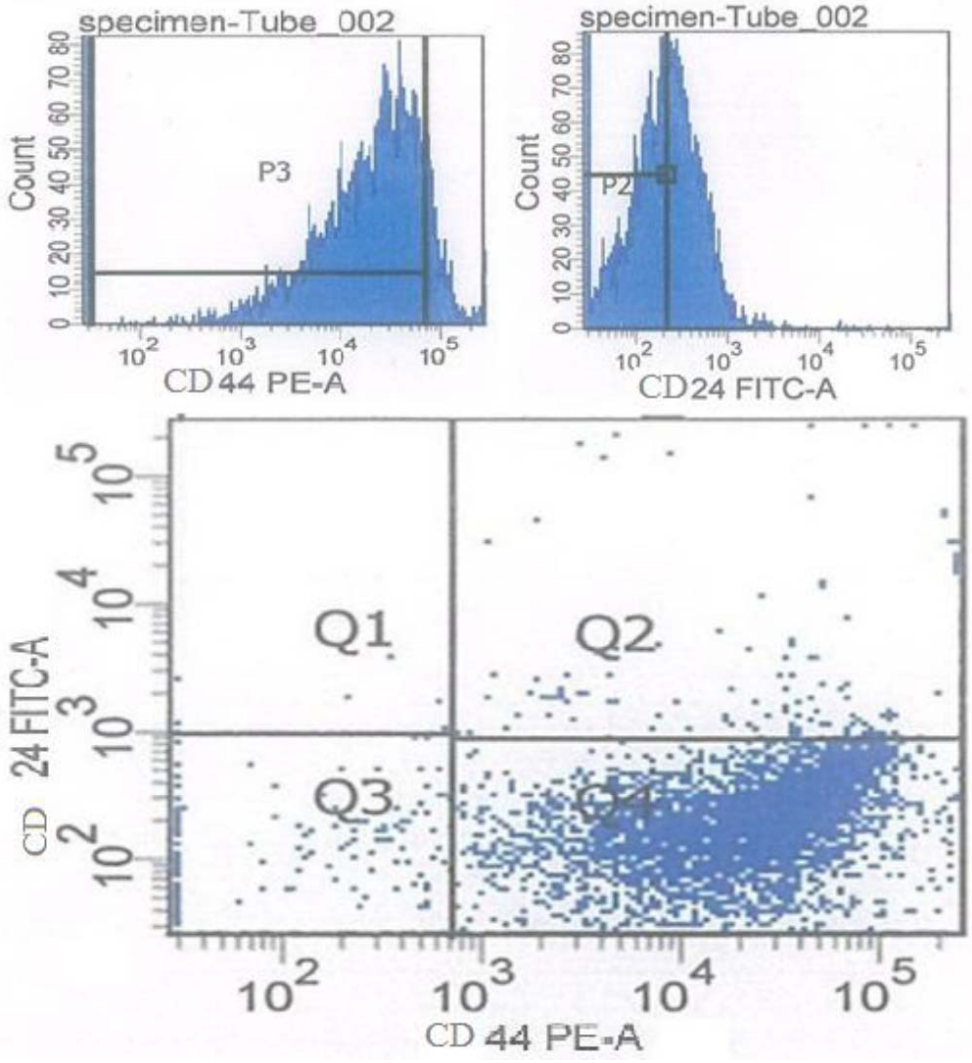

Figure 2: Flow cytometry characterization.

Flow cytometry sorting of OCSCs with CD44 and CD24 markers by fluorescence-activated cell sorting, showing 78.9% of OCSCs characterized with CD44+high/CD24-low and 93.3% of cells expressed with CD44+. The majority of cells expressed CD44+/CD24-, which are considered as putative cancer stem cells.

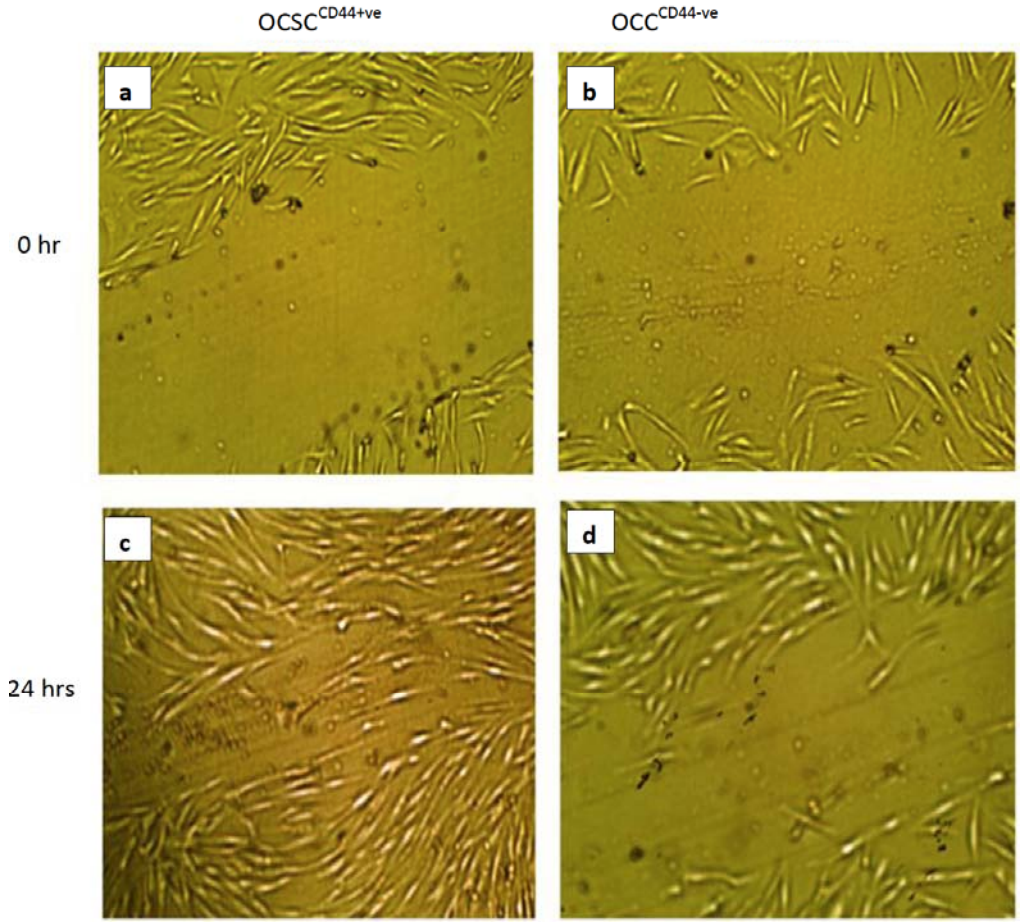

Figure 3: Wound healing assay.

OCSC$^{CD44+ve}$ recovered 'scratch' after 24 hours more efficiently than OCC$^{CD44-ve}$. (**a**) OCSC$^{CD44+ve}$ at 0 hour; (**b**) OCC$^{CD44-ve}$ at 0 hour; (**c**) OCSC$^{CD44+ve}$ at 24 hours; (**d**) OCC$^{CD44-ve}$ at 24 hours; magnification 100X (inverted microscope). *Ovarian Cancer Stem Cells (OCSC); Ovarian Cancer Cells (OCC).*

Wound Healing Assay

Cell mobility is a main factor involved in cancer metastasis [21]. To detect mobility and migration of OCSC$^{CD44+ve}$ versus OCC$^{CD44-ve}$, both types of cells were studied in a wound healing assay (Figure **3a-d**). Large amounts of OCSC$^{CD44+ve}$ migrated into the scratch, which almost recovered (Figure **3c**), while very few OCC$^{CD44-ve}$ migrated (Figure **3d**). The experiment was repeated 3 times with comparable results.

Real-Time qPCR Analysis

Table **1** shows the primer sequence of stemness genes. Expression of self-renewal genes specific to embryonic stem cells was detected in OCSC$^{CD44+ve}$. RNA from OCSC$^{CD44+ve}$ was analyzed by real-time qPCR for Nanog, Oct4, Sox2, and Nestin in comparison with OCC$^{CD44-ve}$ cells and OCs. OCSC$^{CD44+ve}$ overexpressed each stem cell marker versus OCC$^{CD44-ve}$ cells and OCs (Figure **4**).

DISCUSSION

Characterization of the ovarian CSCs is important for future *in vivo* studies with subsequent target therapy for preclinical studies. Most studies lack accuracy in using well-established OCSCs that have passed many sub-cultures. The CSCs may no longer resemble tumor from which they were primarily isolated. In our study we applied the most effective procedure for establishment of OCSCs from solid tumor. These cells were freshly isolated from epithelial ovarian serous carcinoma tumor by enzymatic digestion and mechanical disruption [21]. CD44^{+ve} cells specifically were isolated by anti-CD44 magnetic microbeads. CD44 has been widely demonstrated as a marker that can be elevated in CSCs subpopulation within solid tumor, and its specific association with ovarian CSCs has been frequently reported [22]. We have reported the cluster-forming ability of OCSC$^{CD44+ve}$ on its initial state in culture. Mature or differentiated cells are not capable of forming clusters, which have been indicated in OCC$^{CD44-ve}$. Also

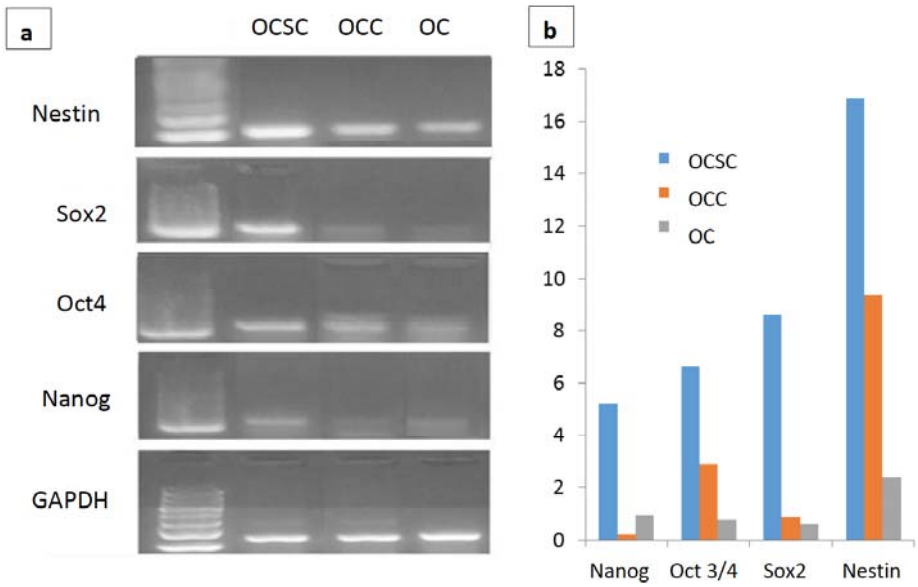

Figure 4: The OCSC$^{CD44+ve}$ have characterization of stem cell marker genes.

(**a**) OCSC$^{CD44+ve}$ overexpressed Nestin, Sox2, Oct4, Nanog when analyzed by real-time qPCR, compared with OCC$^{CD44-ve}$ cells and OC. GAPDH was used as an internal control. (**b**) Average relative expression levels (units) for Nanog, Oct4, Sox2, and Nestin normalized to GAPDH in OCSCs as compared to OCCs and OCs. *Ovarian Cancer Stem Cells (OCSC); Ovarian Cancer Cells (OCC); and Ovarian Cells (OC).*

OCSC$^{CD44+ve}$ have exhibited clear spindle shapes having migratory and invasive properties by undergoing the epithelial-mesenchymal transition (EMT) process, which plays an essential role in the initiation of metastasis for cancer progression [23].

Flow cytometry characterization of OCSCs with CD44 and CD24 markers by fluorescence-activated cell sorting showed 78.9% of OCSCs characterized with CD44+high/CD24-low and 93.3% of cells expressed with CD44+. The majority of cells expressed CD44+/CD24-, which are considered as putative cancer stem cells [24, 25].

Cell mobility is a main factor that drives cancer metastasis [21]. Indeed, OCSC$^{CD44+ve}$ showed higher motility and migration ability than OCC$^{CD44-ve}$ in our wound healing assay. Reports have shown that aggressive characteristics of OCSC$^{CD44+ve}$ may contribute to ovarian cancer pathogenesis [26].

The quantitative analysis by real-time PCR shows that OCSC$^{CD44+ve}$ overexpressed the stemness marker genes *Nanog*, *Oct4*, *Sox2*, and *Nestin* when compared with OCC$^{CD44-ve}$ as positive control and OCs as negative control.

CONCLUSIONS

We have demonstrated that CD44 in malignant ovarian tumors is a critical molecule that exhibits cancer stemness properties that might enhance tumorigenicity and cancer metastasis. CD44 phenotype of ovarian cancer cells has characterized self-renewal marker genes that drive the special properties of CSCs. Our results provide a better understanding of ovarian CSCs, which is important for future in vivo studies with subsequent target therapy for preclinical studies.

CONFLICTS OF INTEREST

The authors have no conflicts of interest to report.

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