

Mitochondrial DNA Deficiency in Ovarian Cancer Cells and Cancer Stem Cell-like Properties

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Abstract. *Background:* A low quantity of mitochondrial DNA (mtDNA) is a risk factor in a variety of tumor types. However, it is unclear how mtDNA reduction influences tumor behavior. *Material and Methods:* mtDNA-deficient ovarian cancer cells were established by ethidium bromide (EtBr) treatment with additive combination of pyruvate and uridine. *Results:* The mtDNA-deficient cells had a low growth and colony-forming efficiency compared to the control cells. RNA sequencing revealed down-regulation of mitochondrion-related genes and up-regulation of genes related to cell proliferation and anti-apoptosis. The expression of genes involved in cancer metastasis, proliferation, angiogenesis, drug resistance and cancer cell stemness were also up-regulated. Intriguingly, cancer stem cell markers CD90 and CD117 were both up-regulated by EtBr dose-dependently in both cell lines. *Conclusion:* MtDNA deficiency may induce ovarian cancer stem cell-like properties through different ways in vitro, therefore contributing to different tumor behaviors.

Patients with ovarian cancer have a high mortality rate, which is mainly due to distant metastasis, chemoresistance and cancer relapse (1, 2). Cancer stem cells (CSCs) are a

sub-population of tumor cells with the properties of self-renewal and tumorigenicity. Evidence has accumulated that CSCs play a key role in tumor metastasis, chemoresistance and relapse (3, 4), and they have therefore become a promising target for cancer treatment (5).

Mitochondria are the site of oxidative phosphorylation, a process by which most of the cell's energy supply of adenosine triphosphate (ATP) is generated by aerobic respiration in the presence of oxygen. Human mitochondrial DNA (mtDNA) is a 16.6-kb circular double-stranded DNA containing 37 genes, including two ribosomal RNAs, 22 transfer RNAs and 13 protein-encoding RNAs (6). The mtDNA-encoded proteins are all subunits of respiratory complexes I, III, IV and V, while the subunits of complex II are entirely encoded by nuclear DNA (nDNA) (7, 8). Unlike nDNA, mtDNA exists in each cell, with several hundreds to more than 10 thousand copies. The copy number of mtDNA in the cell is dependent on various internal or external factors associated with ATP demand, e.g. exercise, hypoxia, and steroid hormones stimulation (9).

Dysregulation of genes involved in glucose metabolism is common in a number of pathological conditions, including metabolic diseases, Alzheimer's disease and cancer. Tumor cells often exhibit decreased oxidative phosphorylation, even in the presence of sufficient oxygen, a phenomenon known as the Warburg effect (10). This effect is due to enhanced transcription of glycolytic genes and reduced transcription of tricarboxylic acid cycle genes (11, 12). Both genetic disorders and chemical treatments may drive reduction of mtDNA copy number and lead to insufficient synthesis of respiratory chain complexes (7). Ethidium bromide (EtBr) is known to inhibit mtDNA replication with a negligible effect on nDNA, and therefore is generally used to generate mtDNA-deficient

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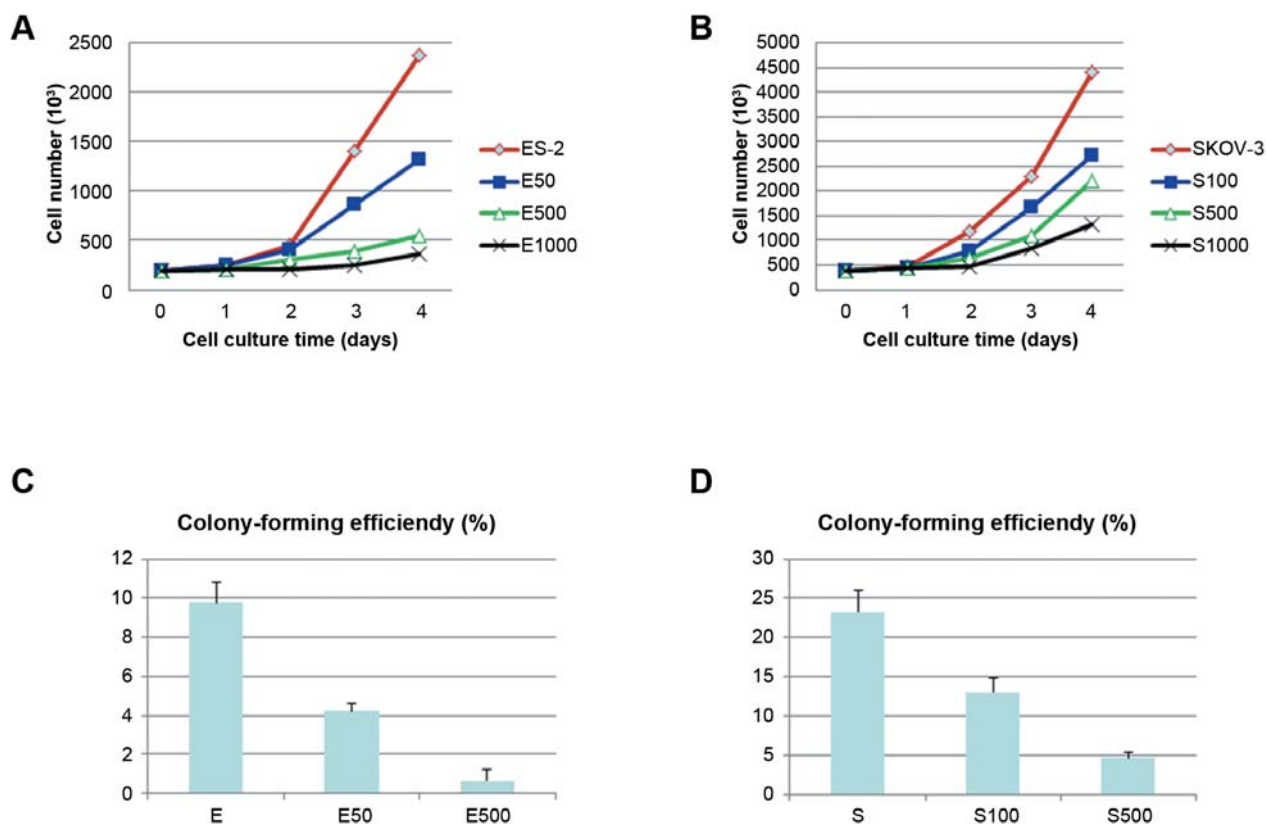


Figure 1. Cell growth curves and histograms of colony-forming efficiency. Both ovarian cancer cell lines ES-2 (A) and SKOV-3 (B) reveal dose-dependent growth inhibition and dose-dependent reduction of colony formation (C and D, respectively) following treatment with ethidium bromide (EtBr). E: ES-2 line; E50, E500, E1000: ES-2 line treated with 50, 500, and 1000 ng/ml of EtBr, respectively. S: SKOV-3 line; S100, S500, S1000: SKOV-3 line treated with 100, 500, and 1000 ng/ml of EtBr. All EtBr-treated cells were cultured under the same concentrations of pyruvate and uridine.

models (7, 13-15). Pyruvate and uridine are nutrients essential for cultured mtDNA-deficient cells to survive (16).

Compelling evidence shows that mtDNA reduction or mtDNA mutation, which may lead to oxidative DNA damage, abnormal expression and mitochondrial dysfunction, is a genetic risk factor for different types of cancers (17-22). Wang and co-workers have found that mtDNA copy number in high-grade tumors is significantly lower than that in low-grade ones. Since the grade of tumor is a crucial prognostic factor, their finding of mtDNA content change in tumor cells may indicate an important genetic event in the progression of ovarian carcinoma (9). However, the biological mechanisms for this phenomenon remain obscure.

In the present study, we aimed to establish ovarian cancer cell line models with reduced mtDNA replication by EtBr treatment and explore the biological changes of these cells compared to those with normal mtDNA replication. Cancer cell stemness properties, including cell proliferation efficiency, and stemness markers in both RNA and protein levels were

investigated. Furthermore, transcriptome changes were explored through high-throughput transcriptome sequencing, some of which were confirmed by other techniques.

Materials and Methods

Cell culture and cell treatment. ES-2 and SKOV-3, obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA) were maintained in our laboratory in Department of Pathology, The Norwegian Radium Hospital, Oslo University Hospital. The ES-2 line was derived from a patient with ovarian clear cell carcinoma, and the SKOV-3 line was derived from malignant ascites of patients with ovarian adenocarcinoma. Both cell lines were cultivated in McCoy's 5A medium (Gibco®, Life Technologies, Oslo, Norway) as ATCC recommends, supplementing with 10% fetal bovine serum (FBS), 100 units/ml penicillin and 100 µg/ml streptomycin. To block mtDNA replication, both cell lines were treated with EtBr at 50 ng/ml, 500 ng/ml, 1,000 ng/ml. To provide intermediate nutrient for EtBr-treated cells (16), 50 µg/ml uridine and 100 µg/ml pyruvate were supplied together with EtBr in the above medium for four days. All cells were incubated at 37°C in a humidified incubator with 5% CO₂.

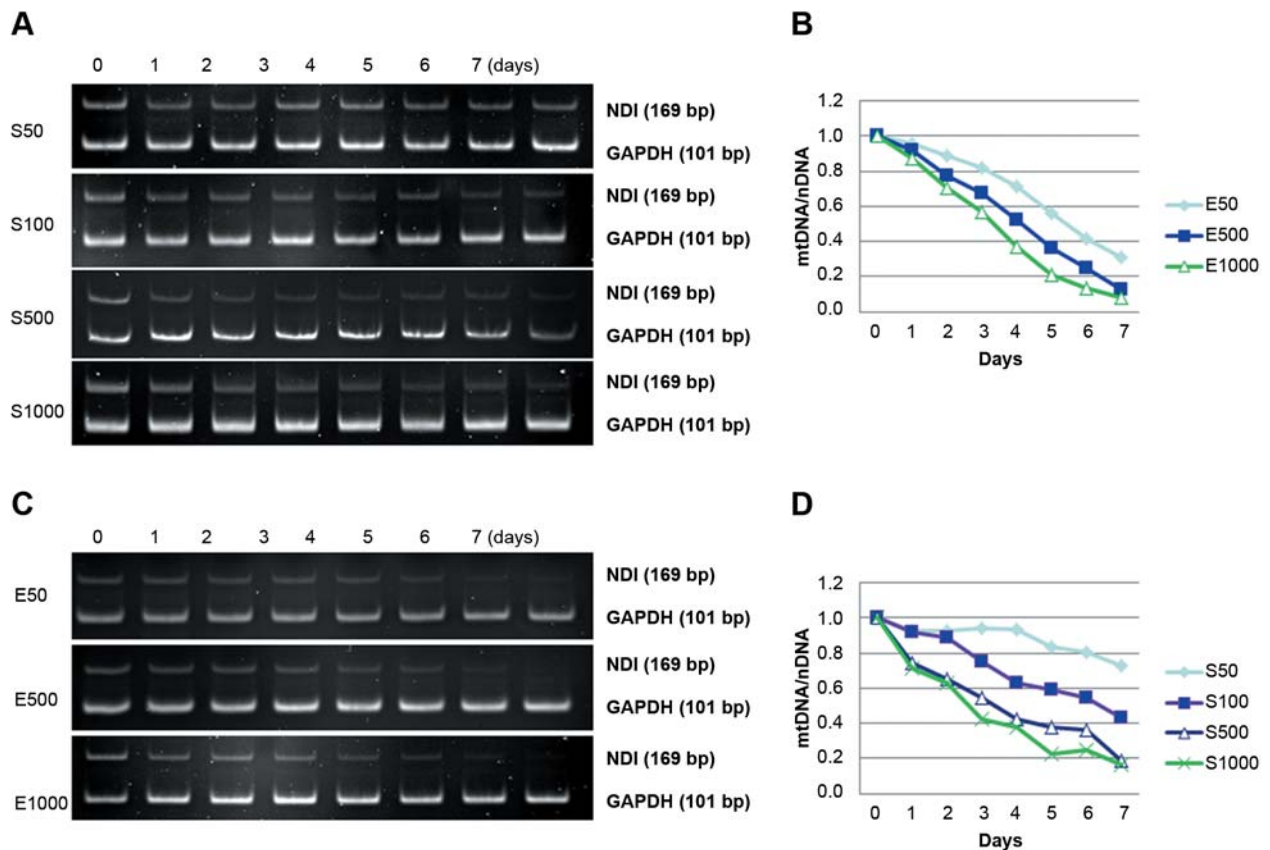


Figure 2. Results of mitochondrial DNA (mtDNA) polymerase chain reaction (PCR) quantification. Representative images of PCR products in ES-2 (A) and SKOV-3 (C) cell lines, respectively. Curves of relative ratio of mtDNA compared to nuclear DNA (nDNA) in ethidium bromide (EtBr)-treated cells (B) and control cells (D) are shown. E50, E100, E500, E1000: ES-2 line treated with 50, 100, 500, and 1,000 ng/ml of EtBr, respectively. S50, S100, S500 and S1000: SKOV-3 line treated with 50, 100, 500, and 1000 ng/ml of EtBr, respectively. All EtBr-treated cells were cultured under the same concentrations of pyruvate and uridine.

Cell counting and cell growth. Every 24 h, cells cultured at different EtBr concentrations were harvested by 0.25% trypsin and EDTA (Invitrogen), resuspended in phosphate-buffered saline (PBS), and counted by Countess[®] Automated Cell Counter (Life Technologies). Cell suspension was gently pipetted up and down several times to avoid cell aggregation before counting cells. Single-cell suspension (10 μ l) was mixed well with 10 μ l of 0.4% trypan blue dye then 10 μ l of the mixture was loaded onto a cell-counting chamber and the chamber was placed in the Countess[®] Automated Cell Counter (Life Technologies) for cell counting. Counted single cells were resuspended at 200 cells/well for the ES-2 line and 400 cells/well for SKOV-3 line in McCoy's 5A medium (Life Technologies) with different concentrations of EtBr in six-well plates and cells were harvested every 24 h for cell counting with the above procedure. This was replicated three times, and the mean values of cell numbers were obtained to derive the cell growth curve.

Colony-forming assay. Counted single cells were resuspended at 200 cells/well for the ES-2 line and 400 cells/well for the SKOV-3

line in McCoy's 5A medium (Life Technologies) with different concentrations of EtBr in six-well plates for 14 days. The cells were gently washed with PBS and fixed with 4% buffered formalin for 15 min before staining with 1% crystal violet for 30 min. The plates were then gently washed with PBS and dried in air. Colonies were evaluated under microscopy and those with more than 30 cells were considered valid as colonies. Colony-forming efficiency was defined as valid colonies/input cells \times 100%. Data are representative of three independent experiments.

DNA and RNA preparation. Total genomic DNA (gDNA), which includes nDNA and mtDNA, was extracted from approximately 10^6 cells using PureLink[™] Genomic DNA Mini Kit (Invitrogen), and total RNA was extracted from the same number of cells using RNeasy Micro kit (Qiagen, Hombrechtikon, Zürich, Switzerland), according to the instruction manuals. DNA and RNA quality and quantity were assessed by NanoDrop[®] ND-1000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA). RNA quality was considered to be good when the ratio of optical density (OD) at 260/280 nm was 1.8 to 2 and OD 260/230 nm was 1.8 or more.

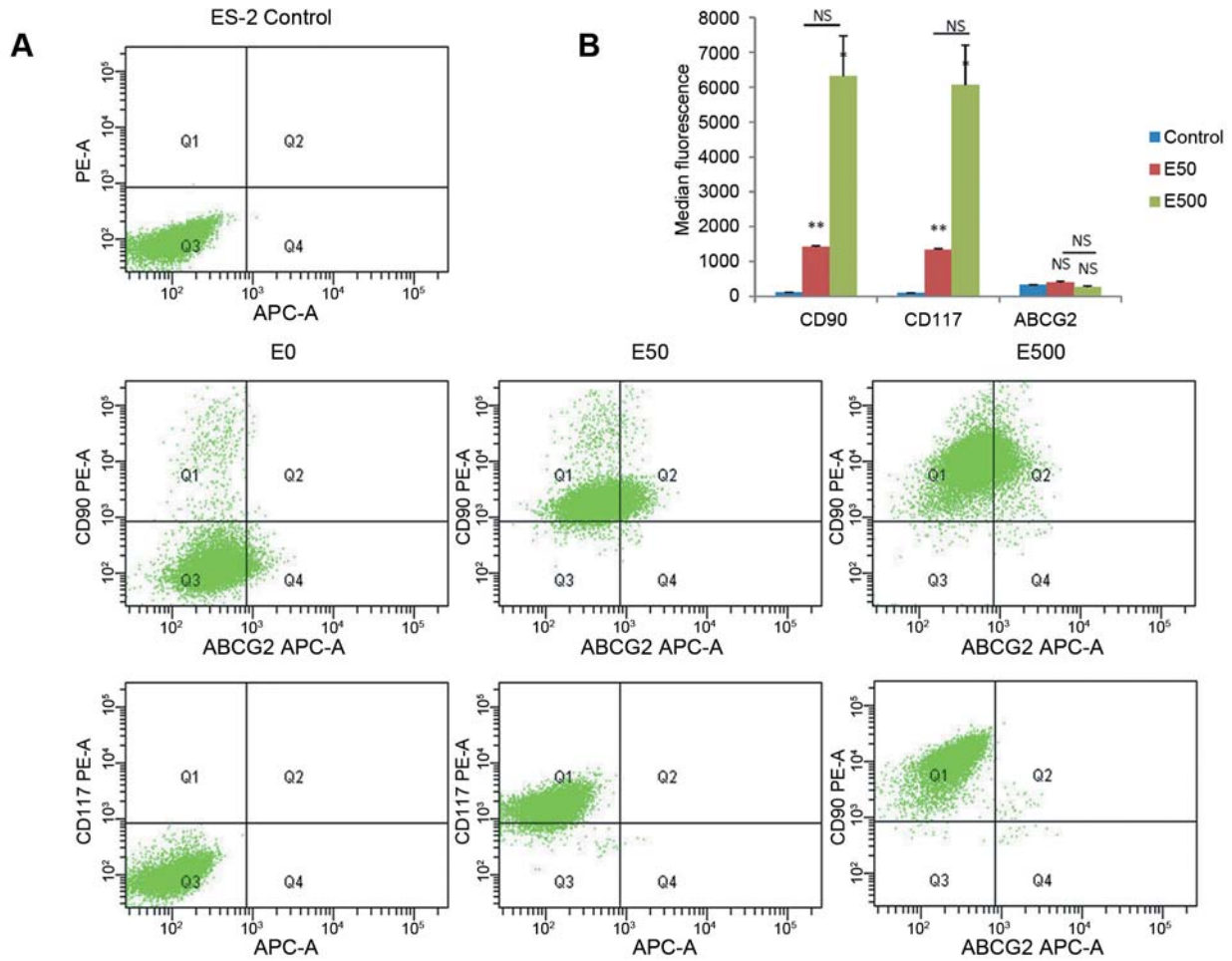


Figure 3. Differential expressions of stem cell surface markers Thy-1 cell surface antigen (THY-1/CD90), v-kit Hardy-Zuckerman 4 feline sarcoma viral oncogene homolog (v-kit/C-Kit/CD117) and ATP-binding cassette, sub-family G, member 2 (ABCG2) in ES-2 cell line. Representative flow cytometric results (A), and corresponding histograms of the fluorescence intensities detected by flow cytometry (B). Both CD90 and CD117 expressions were significantly up-regulated in ethidium bromide (EtBr)-treated ES-2 cells compared to control cells, and the differences between cells treated with different concentrations of EtBr and the controls were significant according to statistical analyses as shown in B. The expression of ABCG2 was not significantly changed. E50, E500: ES-2 line treated with 50, and 500 ng/ml of EtBr. * $p < 0.05$; ** $p < 0.01$. NS: Not significantly different, $p > 0.05$. All EtBr-treated cells were cultured under the same concentrations of pyruvate and uridine.

mtDNA quantification. Total gDNA was amplified by polymerase chain reaction (PCR) to obtain the relative ratios of mtDNA to nDNA. The primers for mitochondrial gene mitochondrially encoded NADH dehydrogenase 1 (*ND1*) were: forward 5'ACTA CAACCCTTCGCTGACG 3' and reverse 5'GCCTAGGTTGA GGTTGACCA 3', with product length of 169 bp. The primers for nuclear gene glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) were: forward 5'CCTCAAGATCATCAGCAATGC3' and reverse 5' TGGTCATGAGTCCTTCCACG3', with product length of 101 bp. Primers were optimized to avoid across interactions. One nanogram of gDNA was added to the PCR system for *ND1* and *GAPDH* amplification simultaneously under the following PCR program: initial denaturation at 95°C for 10 min; followed by 35 cycles of 95°C for 15 seconds, 57°C for 30 sec and 72°C for 30 sec; then 75°C for 10 min and held at 4°C.

PCR product (4 μ l) was then well mixed with 5 μ l diethylpyrocarbonate water and 1 μ l 10 \times Blue Juice™ gel loading buffer (Invitrogen) and subsequently applied to 7.5% polyacrylamide gel electrophoresis. A 50 bp DNA ladder was used to confirm the correct band. The gel was incubated in EtBr Buffer for 10 min before being exposed to G:Box imaging system (Syngene, Cambridge, United Kingdom). Quantity One software (version 4.3; Bio-rad Laboratories, Hercules, CA, USA) was used to analyze the quality and the quantity of the bands detected.

Flow cytometry. Approximately 10⁶ cells for each sample were collected and resuspended in 3 ml ice-cold PBS in Falcon® tubes for investigation of cell surface markers Thy-1 cell surface antigen (THY-1/CD90), v-kit Hardy-Zuckerman 4 feline sarcoma viral oncogene homolog (v-kit/C-Kit/CD117) and ATP-binding

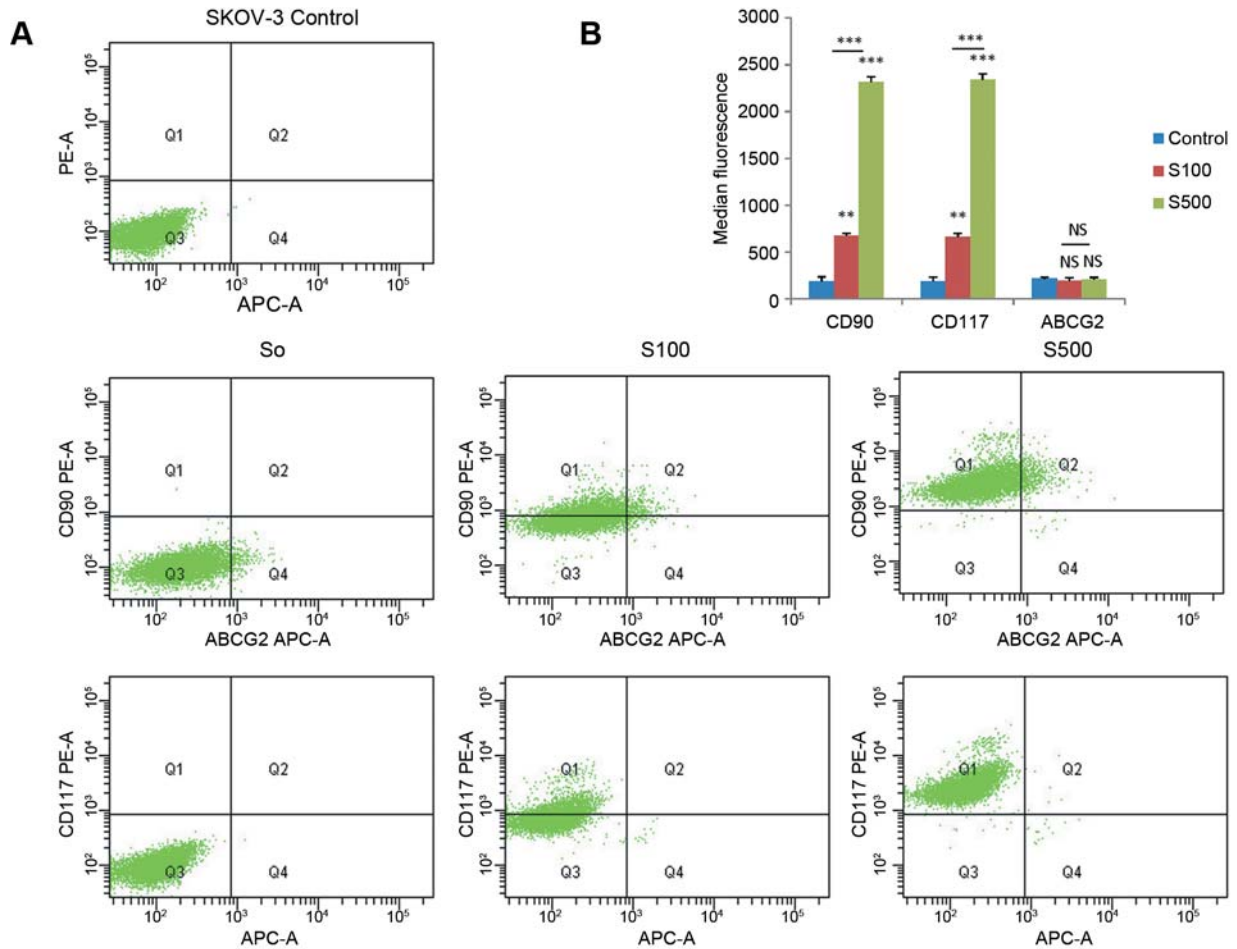


Figure 4. Representative flow cytometric results (A), and corresponding histograms of the fluorescence intensities detected by flow cytometry (B). Both CD90 and CD117 expressions were significantly up-regulated in ethidium bromide (EtBr)-treated SKOV-3 cells compared to control cells, and the differences between cells treated with different concentrations of EtBr and the controls were significant according to statistical analyses as shown in B. The expression of ABCG2 was not significantly changed. S100, S500: SKOV-3 line treated with 100, and 500 ng/ml of EtBr, respectively. ** $p < 0.01$; *** $p < 0.001$; NS: not significantly different, $p > 0.05$. All EtBr-treated cells were cultured under the same concentrations of pyruvate and uridine.

cassette, sub-family G, member 2 (ABCG2). Monoclonal antibodies to CD90 and CD117 directly conjugated with phycoerythrin (PE) and monoclonal antibody to ABCG2 directly conjugated with allophycocyanin (APC) were obtained from BD Bioscience (San Jose, CA, USA). The cells were twice washed with ice-cold PBS and incubated in optimized dilutions of the above antibodies in the dark for 30 min. The cells were then filtered in a 35- μ m nylon mesh cell strainer cap (BD Pharmingen Company, Roskilde, Denmark) immediately before being applied to a flow cytometer (BD™ LSRII yellow laser, BD Bioscience, CA, USA). PE mouse IgG2b and APC mouse IgG2b isotype controls, both obtained from BD Pharmingen Company, were used for negative controls. For each cell sample, variable and single cells were gated before fluorescence was analyzed. FlowJo (version 10.0.6, FlowJo, Ashland, Oregon, USA) was used to analyze the data. The experiments were repeated at least three times, and statistical analyses were performed based on the fluorescence intensity values.

RNA sequencing. After total RNA extraction and DNase treatment, magnetic beads with Oligo (dT) (Life technologies, NY, USA) were used to isolate mRNA. The mRNA was fragmented into short fragments by fragmentation buffer then the mRNA fragments were used as templates to synthesize cDNA. Short fragments were purified and resolved with EB buffer for end reparation and single nucleotide adenine addition. After that, the short fragments were connected with adapters. After agarose gel electrophoresis, the suitable fragments were selected for the PCR amplification as templates. During the quality control steps, Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA) and ABI StepOnePlus Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) were used in quantification and qualification of the sample library. Finally, the library was sequenced using Illumina HiSeq™ 2000 (Illumina, San Diego, CA, USA).

RNA-sequencing data analysis. RNA-sequencing experiments were performed at the Beijing Genomics Institute, Hong Kong. Data

filtering and quality control of raw sequencing data, alignment of raw sequence reads to human reference genome HG19 and genomic annotations were carried out at the Beijing Genomics Institute according to a previous publication (23). In total genomic reads, there were 39693774 (77.31%), 41280763 (77.25%), 43254963 (78.26%) and 39800036 (77.39%) unique matches in sample ES-2 line control (E0), ES-2 line treated with 500 ng/ml of EtBr (E500), SKOV-3 line control (S0) and SKOV-3 line treated with 500 ng/ml of EtBr (S500), respectively. Gene expression level was calculated by the reads per kilobase transcriptome per million mapped reads method (RPKM) (23). Subsequently, differential gene-expression analysis between the samples was carried out. The distributions were assumed to be normal when the difference between the log-transformed RPKM levels of S0/E0 and S500/E500 samples was assessed. Genes with a difference in expression significant at a p-value of less than 0.01 were defined as being significantly differentially expressed between the two groups (24). Based on the selected significantly differentially expressed genes, Gene Ontology (GO) functional annotation was performed by DAVID tool (25). Finally, selected genes highly enriched in certain functional annotation categories were depicted in color-coded heat maps, where red and green represent up-regulation and down-regulation, respectively.

Western blotting. Cells were harvested by 0.25% trypsin and EDTA (Invitrogen) and rinsed twice with ice-cold PBS. Total proteins were obtained with our optimized procedure (26) and measured by the Bio-Rad protein assay. Equal amounts of proteins from each sample in sodium dodecyl sulfate loading buffer was boiled for 5 min and subjected to 10% polyacrylamide gel electrophoresis and then electrotransferred to high-quality polyvinylidene difluoride membrane in a Trans-Blot apparatus (Bio-Rad Laboratories). The membrane was blocked with 5% fat-free milk for 1 h at room temperature and incubated overnight at 4°C with primary antibodies at optimized concentrations (Table I). After washing with PBS-tween 0.05% (PBST), the blot was incubated with the corresponding secondary antibodies conjugated with horseradish peroxidase (HRP). After several washes with PBST, the blot was visualized using an enhanced chemiluminescence detection kit (Amersham, GE Healthcare, Buckinghamshire, UK) by following the manual. The experiments were performed three times.

Immunocytochemistry (ICC). Paraffin-embedded formalin-fixed cyto blocks of cultured cell samples were prepared with the same procedure as in our previous study (26). And the cyto blocks were cut into 3 µm paraffin sections for ICC. Dako Envision™ FLEX+ system (K8012; Dako, Glostrup, Denmark) and the Dako Autostainer were used according to the manual instructions for ICC. The Paraffin sections were de-paraffinized and epitopes unmasked in pre-treatment module (PT link, Dako, Glostrup, Denmark) with appropriate target retrieval solution (Dako; Table I), and then blocked with peroxidase blocking solution (Dako) for 5 min. The slides were incubated with primary antibodies for 30 min at optimized concentrations (Table I). The slides were then incubated with corresponding secondary antibody for 30 min, followed with mouse linker for 15 min and HRP for 30 min at room temperature. Slides were then stained with 3, 3'-diaminobenzidine tetrahydrochloride (DAB) for 10 min and counterstained with hematoxylin for 20 sec, dehydrated, and mounted in Richard-Allan Scientific Cyto seal XYL (Thermo Scientific, Waltham, MA, USA) before microscopic evaluation.

Table I. Antibody information used for immunocytochemistry (ICC) and western blotting (WB).

Antibody name	Company	Catalog no.	Source	Dilution WB	Dilution ICC	Retrieval solution
VEGFA	Santa Cruz Biotechnology, Dallas, Texas, USA	Sc-507	Rabbit poly-clonal	1:500	1:200	LPH
WEE1	Santa Cruz Biotechnology, Dallas, Texas, USA	Sc-5285	Mouse mono-clonal	1:500	1:300	HPH
HES1	Abcam, Cambridge, UK	Ab87395	Mouse mono-clonal	1:1000	1:100	HPH

LPH: Low pH; HPH: high pH.

Statistical analyses. All the experiments were performed at least three times. Statistical analyses were performed by Student's *t*-test with SPSS software (version 18.0, IBM, Armonk, NY, USA). Statistical significance was considered if $p < 0.05$. Data are shown as The mean ± SD.

Results

Proliferation and clonogenicity were suppressed by EtBr. Cell growth of ovarian cancer cell lines ES-2 and SKOV-3 treated with different concentrations of EtBr (50 ng/ml, 500 ng/ml and 1,000 ng/ml for ES-2; 100 ng/ml, 500 ng/ml and 1000 ng/ml for SKOV-3) was evaluated by cell counting at different time points. For both ovarian cancer cell lines, cells treated with EtBr grew slowly compared to controls ($p < 0.05$), and the higher concentration EtBr was, the more slowly ovarian cancer cells grew (Figure 1A and B).

Both ovarian cancer cell lines were treated with the above concentrations of EtBr for clonogenicity studies. For both cell lines, EtBr-treated cells generated fewer colonies than the corresponding control cells ($p < 0.05$), and the higher the concentration of EtBr was, the fewer colonies were generated (Figure 1C and D).

In both above studies, ES-2 cell proliferation and clonogenicity efficiency was more suppressed than SKOV-3 under the same EtBr concentration. ES-2 seems more sensitive to EtBr than SKOV-3.

EtBr treatment resulted in mtDNA deficiency. Different concentrations (50 ng/ml, 500 ng/ml and 1000 ng/ml) of EtBr were used to treat ovarian cancer cell lines ES-2 and SKOV-3, and mtDNA quantity was examined by PCR analysis. ES-2 line was sensitive and mtDNA was reduced when cells were exposed to 50 ng/ml and higher concentrations of EtBr (Figure 2C and D). However SKOV-

3 line was not as sensitive as ES-2 line to 50 ng/ml of EtBr, hence 100 ng/ml of EtBr was used for the SKOV-3 line instead of 50 ng/ml for ES-2 line (Figure 2A and B). The mtDNA quantity of SKOV-3 line decreased when cells were exposed to 100 ng/ml or higher concentrations of EtBr. For both cell lines, the mtDNA quantity was more down-regulated when cells were exposed to higher concentrations of EtBr (Figure 2). It was found that treatment with 500 ng/ml of EtBr for 4 days resulted in cells with significantly lower mtDNA content in both cell lines, which were used for further analyses in our present study.

Expression of stem cell surface markers was up-regulated by EtBr treatment. CD90, CD117 and ABCG2 are frequently used as surface markers for CSCs (27, 28). The expression levels of CD90, CD117 and ABCG2 on ovarian cancer cells treated with different concentrations of EtBr (50 ng/ml and 500 ng/ml for ES-2; 100 ng/ml and 500 ng/ml for SKOV-3) and control cells were evaluated by flow cytometric analysis. A small CD90-positive population was observed in the untreated ES-2 line. For both ES-2 (Figure 3) and SKOV-3 (Figure 4) lines, the proportions of CD90- and CD117-expressing EtBr-treated cells were significantly higher than for untreated control cells ($p < 0.05$), and a higher concentration of EtBr resulted in a significantly higher proportion of the expression ($p < 0.05$). However, no significant difference in these proportions was found for ABCG2 between cells treated with 50 ng/ml or 500 ng/ml EtBr cells and control cells for either cell line ($p > 0.05$).

Transcriptome changes in mtDNA-deficient cells. Transcriptome changes of ovarian cancer cell lines ES-2 and SKOV-3 treated by 500 ng/ml EtBr compared to controls were evaluated by RNA-sequencing. After EtBr treatment, certain genes responding to DNA-damaging agents, such as protein phosphatase 1 regulatory subunit 15A (*PPP1R15A*), were significantly up-regulated in both cells lines. GO functional analysis showed that expression of 19 mitochondrion-related genes (Figure 5A) was significantly attenuated in both cell lines by EtBr treatment ($p < 0.05$), indicating inhibition of oxidative phosphorylation.

According to GO analysis, the regulation of cell proliferation, which involved 20 genes, including prostaglandin-endoperoxide synthase 2 (*PTGS2*), interleukin 6 signal transducer (*IL6ST*), jagged 1 (*JAG1*), interleukin 11 (*IL11*), hes family bHLH transcription factor 1 (*HES1*), vascular endothelial growth factor A (*VEGFA*), activating transcription factor 3 (*ATF3*), interleukin 1 beta (*IL1B*) and ATP-binding cassette, sub-family C member 3 (*ABCC3*) (Figure 5B), was significantly up-regulated ($p < 0.05$).

Ten anti-apoptotic genes were significantly up-regulated based on GO analysis (Figure 5C; $p < 0.05$). Furthermore, WEE1 G2 checkpoint kinase (*WEE1*) gene expression was up-regulated, which in cancer is believed to negatively

regulate DNA damage-induced apoptosis (29), and is associated with poor prognosis in ovarian carcinoma (30).

In addition, some other genes closely associated with stem cell maintenance were found to be up-regulated, including growth differentiation factor 15 (*GDF15*) (31) and BMP and activin membrane-bound inhibitor (*BAMBI*) (32) in the list of differentially expressed genes (Figure 5D). To assess whether the differential expression of some genes found by RNA sequencing was reflected in the level of protein expression, three gene products namely VEGFA, WEE1 and HES1, were measured by western blotting and ICC. These three markers showed increased expression in EtBr-treated cells for both cell lines in contrast with the controls (Figure 6) verified by both techniques, which is qualitatively in accordance with the RNA sequencing results.

Discussion

CSCs are a sub-population of tumor cells that may stay dormant in an appropriate niche where they are not recognized by current chemotherapy and other antitumor therapies (33-35). However, under specific conditions, they are activated and recruited into different tissues, where they play key roles in chemoresistance, relapse and metastasis (36, 37). Due to these properties, CSCs are thought to be promising targets in preventing cancer relapse and vastly improving cancer survival probability (34), although it is still such a challenge to target them due to their complex biology and unstable status (38, 39).

In the present study we found ovarian cancer cells became more dormant stem-like cells by EtBr treatment. When treated with EtBr, both ovarian cancer cell lines ES-2 and SKOV-3 generated relatively quiescent and low-proliferating characteristics compared to control cells, and this is coincident with the dormancy probability of CSCs (33, 34). Furthermore, the dormancy of tumor cells may be due to their higher expression of stem cell markers. This phenomenon may have a similar molecular mechanism to that we verified for ovarian cancer cells under hypoxia treatment (40). Ovarian cancer cells treated with hypoxia expressed high levels of the markers of cancer cell stemness and had a low proliferation and colony-forming efficiency, but significantly highly proliferative and aggressive properties were generated after these cells were recultured under normal oxygen conditions (40).

Furthermore, mtDNA in both cell lines was reduced by EtBr, and we propose that aerobic respiration in these cells was suppressed due to deficiency in subunits of respiratory complexes encoded by mtDNA. Cells were, thus, forced to switch to anaerobic respiration to supply ATP and other intermediate molecules. This is in parallel with our finding that hypoxia-related factors were enhanced, including hypoxia up-regulated 1 (*HYOU1*) and VEGFA. Hypoxia is

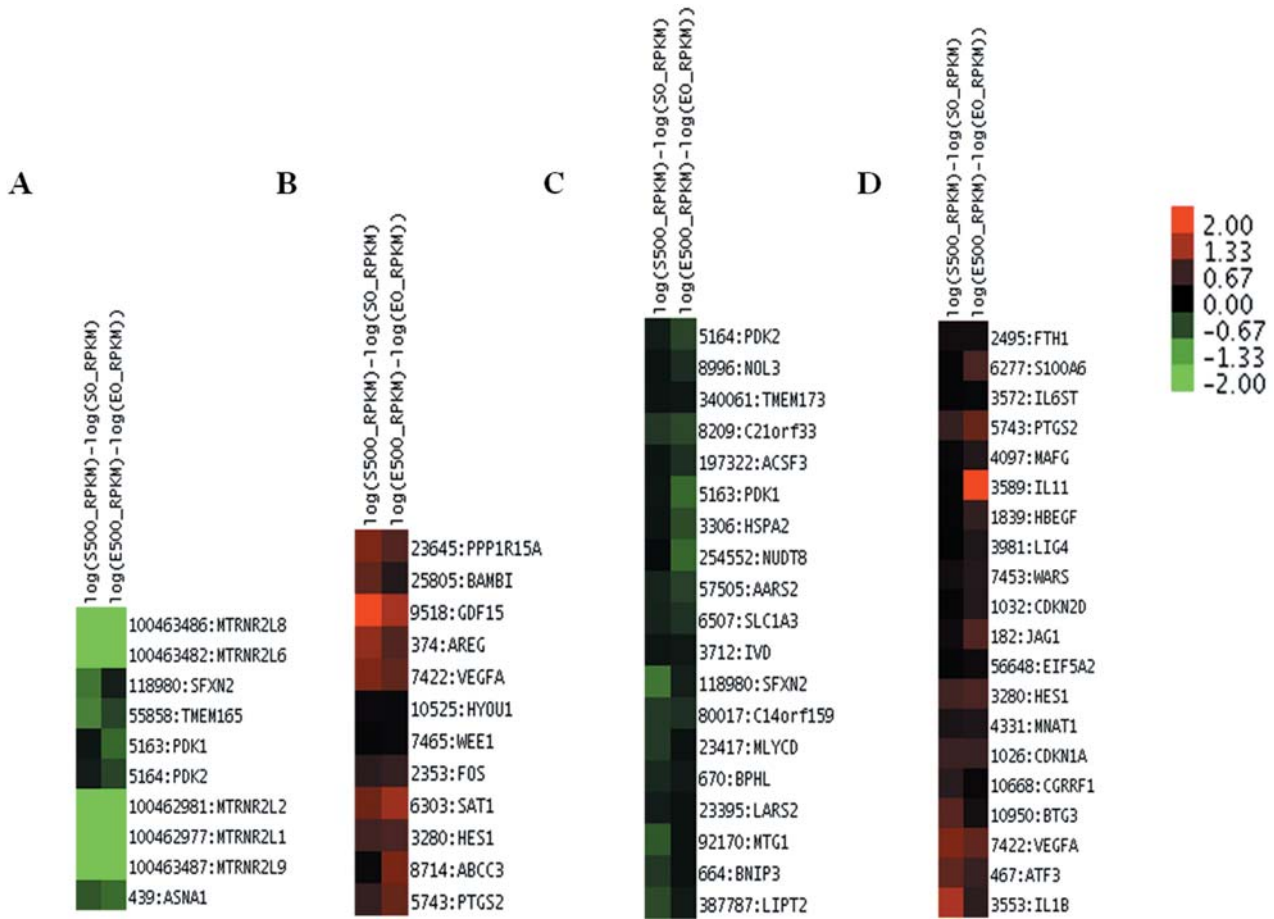


Figure 5. Color-coded heat maps of differentially expressed genes (DEGs) and Gene Ontology (GO) functional annotation. A: GO 0005739, mitochondrion; B: GO 0042127, regulation of cell proliferation; C: GO 0006916, anti-apoptosis; D: selected genes from the DEG list. S500 and E500: SKOV-3 and ES-2 lines cultured in medium containing 500 ng/ml ethidium bromide (EtBr) with the same concentrations of pyruvate and uridine for four days; S0 and E0: SKOV-3 and ES-2 lines cultured in normal medium as control.

one of the key ways to induce CSCs or up-regulate cancer cell stemness (41-43). VEGFA is a key factor of angiogenesis and vascular permeability in tumor, but it was recently found to induce and regulate CSC properties by autocrine stimulation in tumors (44, 45).

To identify putative CSCs, both CSC-specific markers and functional studies are widely used. CSCs may explain the heterogeneity, metastasis, recurrence, chemoresistance and radiation resistance of tumors, and the evasion of apoptosis of CSCs is considered a main mechanism of recurrence and chemoresistance of cancer (46). In the study, cell surface markers of CSCs CD90 and CD117 (47, 48) were found to be expressed in an increasing proportion of cells by EtBr treatment. Nevertheless, we did not observe differential expressions of CD90/THY1 and CD117/c-KIT by RNA-sequencing. The changes at the protein level may be due to post-transcriptional regulation or other unknown mechanisms.

CSC-related genes were up-regulated by EtBr treatment, including angiogenesis factor *VEGFA*, chemoresistance-related factor *ABCC3*, self-renewal and tumorigenicity-related factor *HES1*, and others. The function of VEGFA was recently found not to be limited to angiogenesis and vascular permeability. VEGFA-mediated signaling contributes to the function of CSCs, including tumorigenesis and tumor initiation by autocrine stimulation in tumors (44, 45). HES1, as a transcription factor, was recently found to be involved in self-renewal and tumorigenicity of stem-like cancer cells in colon cancer (49). *ABCC3* and *ABCG2* are two well-known drug transporters of the ABC family which are able to efflux anticancer drugs from tumor cells and thus contribute to chemoresistance (50, 51). In contrast to the increased expression of *ABCC3* in EtBr-treated cells, *ABCG2* was not found to be differentially expressed at the RNA level nor at the protein level in our study. This may reflect a possibility

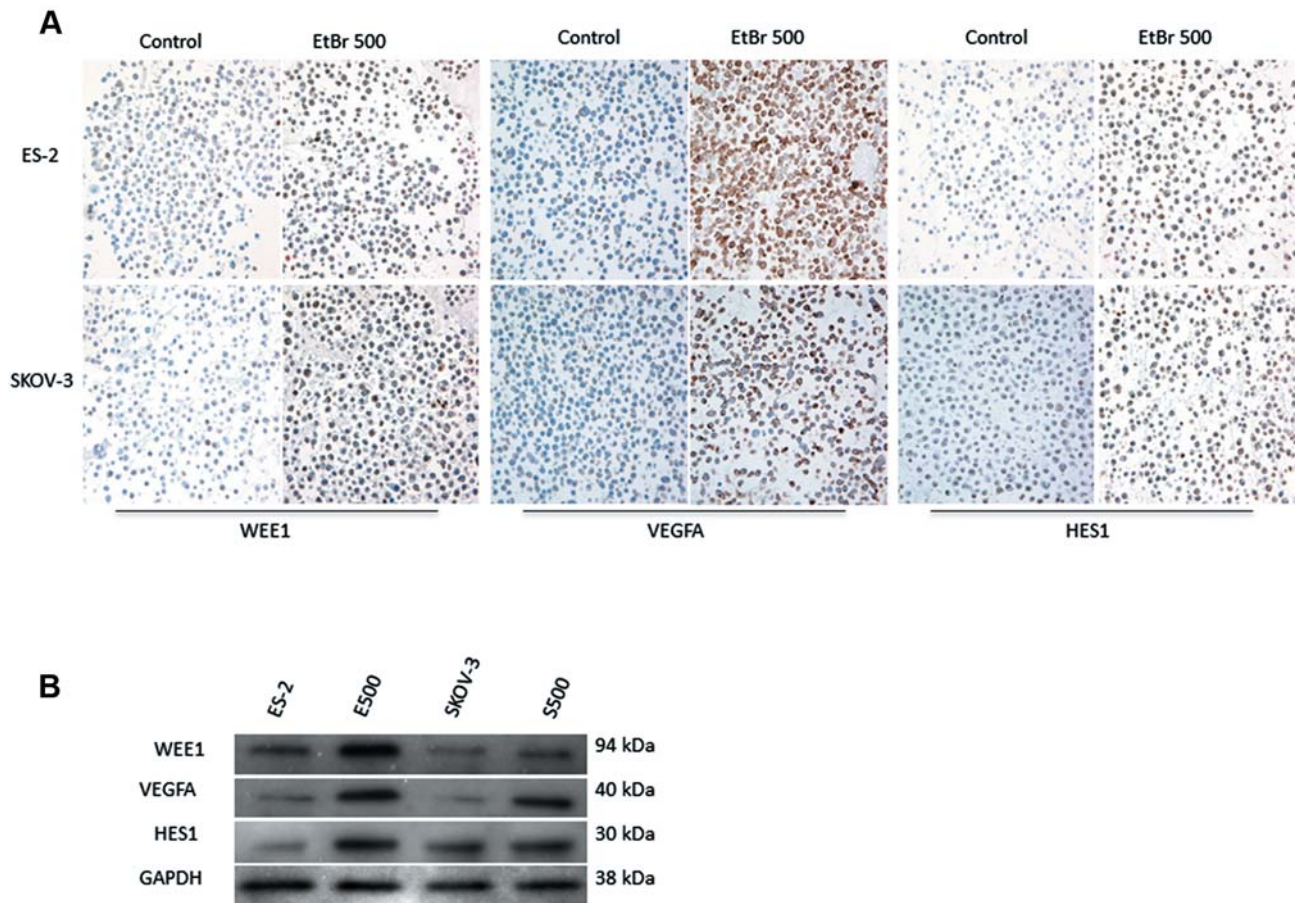


Figure 6. Protein expression was verified in ovarian cancer cell lines ES-2 and SKOV-3 treated with 500 ng/ml ethidium bromide (EtBr) and controls. A: Immunocytochemical study verified that WEE1 G2 checkpoint kinase (WEE1), vascular endothelial growth factor A (VEGFA) and hes family bHLH transcription factor 1 (HES1) were highly expressed after EtBr treatment in both lines. All images were taken at $\times 200$. B: Western blotting shows that the expressions of WEE1, VEGFA and HES1 in EtBr-treated cells was stronger than those in their corresponding controls for both cell lines. E500, S500: Cultural medium containing 500 ng/ml EtBr under the same concentrations of pyruvate and uridine.

that these two molecules work differently, and ABCC3 plays a more important role in typical mtDNA-deficient ovarian cancer cells. Furthermore, among the up-regulated genes, *PTGS2* (52), *ATF3* (53), *HES1* (49, 52), *VEGFA* (44, 45), *IL11* (54), *IL6ST* (54) and *IL1B* (55) have been reported to induce, maintain or enhance cancer cell stemness. ABCC3, as a member of the ABC transporter family, functions to efflux anticancer drugs from tumor cells and thus contributes to multidrug resistance.

Biomarkers for CSCs are not universal, and more and more new markers are being found to identify CSCs. *GDF15* plays a functional role in radioresistance in nasopharyngeal carcinoma (56), and was thought to be related to cancer cell stemness (31). *ATF3* gene can up-regulate transforming growth factor-beta (*TGF- β*) itself and its target genes snail family zinc finger 1 (*SNAIL*), snail family zinc finger 2 (*SLUG*) and twist family bHLH transcription factor 1

(*TWIST*), and enhance epithelial-to-mesenchymal transition and breast cancer cell stemness (53). Fluorouracil-induced increase of colorectal CSCs can be suppressed by the inhibition of *PTGS2* and *NOTCH1/HES1* (52). *IL1B* and *TGF β* cooperate and induce glioma stem cells (55). *IL11*, as an *IL6* family cytokine, plays a stronger role compared to *IL6* in gastrointestinal tumorigenesis, proliferation and invasion (54). *IL11* is the dominant *IL6* family cytokine during gastrointestinal tumorigenesis and can be targeted therapeutically. *BAMBI* plays a key role in maintaining the stemness of mesenchymal stem cells and blocking their differentiation into cancer-associated fibroblasts by the inhibition of *TGF β* signaling (32).

In summary, EtBr treatment results in dose-dependent reduction of mtDNA, and mtDNA-deficient cells were obtained from ovarian cancer cell line ES-2 and SKOV-3 when treated with 500 ng/ml of EtBr for 4 days. The mtDNA-

deficient cells grew slowly, with lower capability in colony formation, a similar finding with tumor cells under hypoxia. Furthermore, these mtDNA-deficient cells expressed highly a series of genes related to anti-apoptosis, regulation of proliferation, and cell stemness. In short, mtDNA-deficient ovarian cancer cells may be ideal models for CSC studies.

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