Isolation and characterization of cancer stem cells from a heterogeneous population in human ovarian cancer

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Isolation and characterization of cancer stem cells from a heterogeneous population in human ovarian cancer

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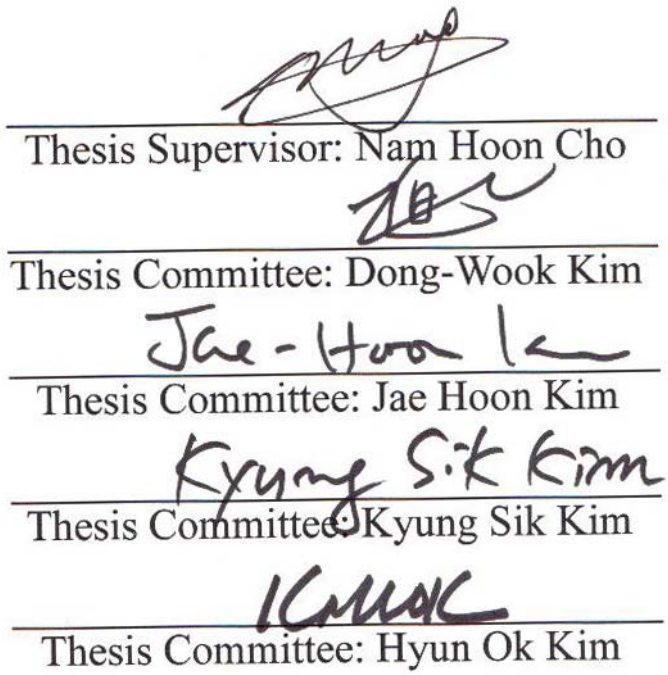
This certifies that the Doctoral Dissertation of Ming Qing Gao is approved.



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ABSTRACT

Isolation and characterization of cancer stem cells from a heterogeneous population in human ovarian cancer

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Cancer stem cells (CSCs) have been identified in solid tumors and cancer cell lines. Here, we isolated a series of cancer cell clones, which were heterogeneous in growth rate, cell cycle distribution, and expression profile of genes and proteins, from ovarian tumor specimens of a patient and identified a subpopulation enriched for ovarian CSCs defined by CD24 phenotype. Experiments in vitro demonstrated $CD24^+$ subpopulation possessed stem cell-like characteristics of remaining quiescence and more chemoresistance compared to $CD24^-$ fraction, as well as a specific capacity for self-renewal and differentiation. In addition, injection of 5×10^3 $CD24^+$ cells was able to form tumor xenografts in nude mice, whereas equal number of $CD24^-$ cells remained nontumorigenic. We also found $CD24^+$ cells expressed higher mRNA levels of

some “stemness” genes, including Nestin, β-catenin, Bmi-1, Oct4, Oct3/4, Notch1, and Notch4 which were involved in modulating many functions of stem cells, and lower E-cadherin mRNA level than $CD24^-$ cells. Altogether, these observations suggest human ovarian tumor cells are organized as a hierarchy and CD24 demarcates an ovarian cancer-initiating cell population. These findings will have important clinical applications for developing effective therapeutic strategies to treat ovarian cancer.

Key words: CD24, Ovarian cancer, Cancer stem cell, Tumorigenicity

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Ⅰ. INTRODUCTION

In spite of advances in the treatment of ovarian carcinoma, it remains the leading cause of death among gynecologic neoplasms because current therapies are limited by the emergence of chemotherapy-resistant cancer cells following the high initial response rate[1, 2]. Understanding the biological mechanisms mediating drug resistance may lead to new therapeutic strategies.

Recently, a cancer stem cell (CSC) hypothesis has proposed that heterogeneous tumors contain a small fraction of stem-like cancer cells with sufficiently unique properties that would allow them to survive chemotherapy and retain their tumor- initiating potential [3-5]. These cells are named “CSCs” because they possess properties of keeping quiescent, self-renewal, and

differentiation like normal stem cells. CSCs have been discovered in a large series of tumors including breast[6], prostate[7], pancreas[8], and melanoma tumors[9] since they were first identified in acute myeloid leukemia as possessing the cell surface antigen phenotype $CD34^{+}CD38^{-}$[10]. A significant effort is underway to identify both CSC-specific markers and the tumorigenic potential of these cells.

The existence of CSCs in ovarian cancer was first proven in multilayered spheroid cells isolated from patient ascites[11] and subsequently verified in a mouse model using side population phenotype[12]. Until now, CD133[13, 14], CD44/CD117[15] and CD44/MyD88[16] have been used to identify ovarian CSCs. CD24, a cell surface molecule, appears to be highly expressed in a large variety of human cancers. Recent studies have suggested that CD24 might be a marker for CSCs with specific regards to the breast[6] and the pancreas[8]. It should be noted that it is the absence of CD24, combining the presence of CD44 and EpCAM, that associates with breast cancer stem cells, while Li and colleagues suggested that $CD44^{+}CD24^{+}EpCAM^{+}$ pancreatic cancer cells possessed stem cell properties. This two-edged role of CD24 in the identification of CSC attracts our more interest in the following research.

In the current investigation, we isolated cancer cells from human ovarian tumor specimens and defined the heterogeneous characteristics of these cell clones and identified the potential ovarian CSCs. We revealed that a subset of $CD24^{+}$ cells in ovarian cancer possess CSCs properties of relative quiescence,

self-renewal and differentiation, chemoresistance, and tumorigenicity in nude mice. We also undertook investigation of the possible gene regulations of CD24. We believe this is the first time to isolate CSCs by virtue of CD24 phenotype from human ovarian cancer cells. This identification of ovarian CSCs may provide novel therapeutic approaches to treat ovarian cancer.

Ⅱ. MATERIALS AND METHODS

1. Cell isolation from human ovarian tumors

Tumor tissue specimens categorized as serous or mucinous (cyst) adenocarcinoma were obtained from an ovarian cancer patient during surgical procedure. The study was approved by the local ethics committee and consent was obtained from all patients. Tumor tissues were washed and cut up into small pieces with scissors and then minced completely using sterile scalpel blades, followed by incubation with extracellular matrix degrading enzymes such as dispase, pronase, and DNase (all Sigma, St. Louis, MI, USA) for 12 hours at 37°C. At the end of incubation, cells were filtered through a 100-μm nylon mesh and washed twice with HBSS/2% FBS. To eliminate erythrocytes, dead cells, and other tissue debris, cell suspensions were subjected to ficoll (1.077 g/ml) gradient centrifugation at 400 ×g for 15 min. The cell pellet was collected and suspended in Iscove's modified Dulbecco's medium (IMDM, Gibco BRL, Grand island, NY, USA) containing 100 IU/ml penicillin and 100 μg/ml streptomycin (Gibco BRL), 100 μg/ml gentamicin (Gibco BRL), 2.5 μg/ml amphotericin (Gibco BRL), and 20% FBS. In the following step, cell suspension was seeded in 24-well plates and maintained in a humidified atmosphere of 95% air and 5% CO_2 at 37°C. Tumor cells were separated from fibroblasts by Stable Trypsin-like Enzyme (Gibco BRL) based on the observation that fibroblasts detach earlier from the flask after trypsinzation. Each cell population was

periodically recloned by plating single cells in medium under direct vision and expanding fresh populations from a single clone. Once isolated and recloned, each clone was examined for expression of stem cell markers to determine potential surface markers for ovarian CSCs.

2. RNA extraction and RT-PCR

RNA was extracted from cells using an Ultraspec^{TM}– Ⅱ RNA Kit (Biotecx Laboratories, Inc., Houston, TX, USA) according to the manufacturer's protocol. Complementary DNA was synthesized using 0.5-2 μg of total RNA with SuperScript™ Ⅲ Reverse Transcriptase kit (Invitrogen, Carlsbad, CA, USA), and 2 μl cDNA product was amplified by RT-PCR. GAPDH was used as an internal control in all reactions. PCR products were separated on 2% agarose gel and stained with ethidium bromide. Primers, product size, and PCR conditions are listed in Table 1.

Table 1 Primers, product sizes, and PCR conditions

Molecules	F/R*	Sequence (5'~3')	Size (bp)	Annealing Temperature T	PCR conditions†
CD133	F	TCTCTATGTGGTACAGCCG	350bp	55.5℃	95℃ 15min 94℃ 40sec T ℃ 40sec 72℃ 1min 35 cycles 72℃ 10min
	R	TGATCCGGGTTCTTACCTG			
ABCG2	F	GGGTTCTCTTCTTCCTGACGACC	389bp	62℃	
	R	TGGTTGTGAGATTGACCAACAGAC			
Nestin	F	CCAGAAACTCAAGCACCAC	398bp	62.2℃	
	R	TTTTCCACTCCAGCCATCC			
E-cadherin	F	GCCAAGCAGCAGTACATTCTACACG	342bp	62.7℃	
	R	GCTGTTCTTCACGTGCTCAAAATC			
β-catenin	F	ACTACCACAGCTCCTTCTCTGAG	447bp	61.5℃	
	R	GATAGCGTGTCTGGAAGCTTCCT			
Bmi-1	F	GGAGACCAGCAAGTATTGTCCTATTTG	370bp	62.7℃	
	R	CATTGCTGCTGGGCATCGTAAG			
SMO	F	ATCTCCACAGGAGAGACTGGTTCGG	263bp	62.2℃	
	R	AAAGTGGGGCCTTGGGAACATG			
CD24	F	ACCCAGCATCCTGCTAGAC	287bp	57.5℃	
	R	CTTAAGAGTAGAGATGCAGAA			
CD44	F	AACCTGCCGCTTTGCAGGTGT	342bp	57.5℃	
	R	ATCAAAGGCATTGGGCAG			
CD117	F	GCCCACAATAGATTGGTATTT	570bp	62.2℃	
	R	AGCATCTTTACAGCGACAGTC			
CK18	F	GAGATCGAGGCTCTCAAGGA	357bp	61.5℃	
	R	CAAGCTGGCCTTCAGATTTC			
GAPDH	F	ACAACTTTGGTATCGTGGAA	458bp	57.1℃	
	R	AAATTCGTTGTCATACCAGG			
ESA	F	GGGACCCTGACAGTGGAGCA	410bp	64.0℃	95℃ 15min 94℃ 40sec T ℃ 1min 72℃ 90sec 35 cycles 72℃ 10min
	R	ATCTTCTGCTGTTCACGGGC			
Oct3/4	F	GAAGGTATTCAGCCAAACGAC	487bp	58.1℃	
	R	AGGGACCGAGGAGTACAGTG			
Oct4	F	GGGTTCTATTTGGGAAGGTG	219bp	51.9℃	
	R	AGGTTGCCTCTCACTTGGTT			
Notch-1	F	CAGCGCAGATGCCAACATCCAGGACAACAT	429bp	68℃	95℃ 15min 94℃ 40sec 68 ℃ 2min 35 cycles 72℃ 10min
	R	ATATGATCCGTGATGTCCCGGTTGGCAAAG			
Notch-4	F	TATTCTCATTGCCGGAGCCTCTCGGGAGTA	324bp	68℃	
	R	ACCCTCTCCTCCTTGGTTTATGGGCATTTC			
CD34	F	TGGTCTTCTTTGGGTAAACG	592bp		
	R	TCGGAAGAGTACAGGTGAGAG			

* F: Forward primer; R: Reverse primer

† Conditions for CD34 were pre-denaturation at 95℃ for 15min, followed by 94℃ 40sec, 65℃30sec, -0.5℃/cycle, 72℃ 50sec for 20 cycles, followed by 94℃ 40sec, 55℃ 30sec, 72℃ 50sec for 15cycles, with a final 72℃ 10min.

3. Flow cytometry and fluorescence-activated cell sorting

Detached cells were counted and washed twice with HBSS (calcium and magnesium free) containing 2% heat-inactivated FBS (5 min at 300×g), and then resuspended in HBSS/2% FBS at concentration of 10^6 cells/90 μl. Ten μl of FcR blocking reagent (Miltenyi Biotec Inc., CA, USA) was added and incubated at 4°C for 10 min, after which the sample was washed once and resuspended in 100 μl of HBSS/2% FBS. Antibodies were then added and incubated for 20 min on ice in the dark. After washed twice, cells were resuspended in HBSS/2% FBS (2×10^6 cells/ml) containing 1 μg/ml 7-AAD (Sigma). The suspension was filtered through a 70-μm nylon mesh. Samples were analyzed on a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA, USA) or sorted by EPICS ALTRA flow cytometer (Beckman Coulter, Inc., Fullerton, CA, USA). Side and forward scatter profiles were used to eliminate cell doublets. Dead cells were eliminated using 7-AAD viability dye. Antibodies of anti-CD24- phycoerythrin (PE) or fluorescein isothiocyanate (FITC), anti-CD133-PE, and anti-CD117/c-kit-PE were obtained from BD Pharmingen (San Diego, CA, USA).

4. Cell proliferation assay

Cells were plated in quadruple in 96-well plates and cultured for an appropriate period of time. Proliferation was detected using the Count^{TM} cell viability assay

kit (WelGENE, Seoul, Korea). Cell viability was quantified by measuring the absorbance of the samples with a VersaMax™ microplate reader at a wavelength of 450 nm, and nonspecific readings were eliminated by using a wavelength of 690 nm.

5. Chemotherapeutic sensitivity assay

To assess the chemosensitivity of sorted cells to cisplatin, cells were diluted to 2000 viable cells/100 μl IMDM with 20% FBS, seeded in quadruple to a 96-well ultra low attachment microplate (Costar, Cambridge, MA), and allowed to attach and grow for 48 hours before being exposed to 0, 5, 10, 20, 40, 60, 80, 100, or 120 μmol/l cisplatin (Sigma). After treatment for 72 hours, relative cell numbers were determined by Count^{TM} cell viability assay kit. The half maximal (50%) inhibitory concentration (IC_{50}) value of cisplatin was calculated by SPSS 11.0 as means ± SD.

6. Cell cycle analysis

Flow cytometry analysis of PI-stained cells was performed to demonstrate the progression of the cell cycle. Briefly, 2×10^6 sorted cells were harvested, washed and fixed in 70% ethanol overnight at -20°C. Prior to flow cytometry, cells were washed and stained with 500μl of PI staining solution (50 μg/ml PI, 0.1% Triton X-100, 0.1 μmol/ml EDTA, and 50μg/ml RNase A). DNA content was

determined with a FACScan flow cytometer and the proportion of cells in a particular phase of cell cycle was determined with ModFit LT software.

7. Xenograft experiment for analyses of tumorigenicity

Nude mice were purchased from Central Lab Animal Inc. (Seoul, Korea) and maintained in accordance with the institutional guidelines of Yonsei University College of Medicine. All animal studies were performed according to approved experimental protocols. To assess tumorigenicity, purified $CD24^+$ and $CD24^-$ cells (5×10^3, N=3) were suspended in 100 μl 1:1serum-free-IMDM/Matrigel (BD Biosciences, San Jose, CA, USA) followed by injection s.c. into the right and left flanks of female nude mice (5-6 weeks old). Tumor formation/growth was assayed weekly as a time course. The tumor-bearing animals were sacrificed at the same time when tumor was large enough for subsequent experiments. Animals with no sign of tumor burden were also examined on necroscopy to confirm that there was no tumor development. Xenograft tumors were resected, fixed in formalin and paraffin sections were made for HE staining or immunohistochemistry staining.

8. Statistical analysis

The results are expressed as means ± SD. All statistical analyses were analyzed using ANOVA (SPSS11.5 software). A $p < 0.05$ was considered to be statistically significant.

Ⅲ. RESULTS

1. Clone derivation with different characteristics

The present culture system gave rise to 20 clones designated C2, C3, C4, C4.2, C5, C5.2, C6, C6.2, C7, C7.2, C8, C9, C10, C11, C12, C13, C13.2, C14, C15, and C20. All these clones could be successfully subcultured. Subsequent experiments were performed on these cells cultured for 5-15 passages. Based on the hierarchy model of tumor and previous progenitor cell origin theory [16], we postulated that these primary cancer clones were heterogeneous and CSCs, if they exist, were possibly found in certain clones.

Although no obvious variation was observed in morphology under optical microscope in these clones, growth rate (Figure 1a) and cell cycle distribution (Figure 1b) were significantly different. Subsequently, we investigated the expression of numerous "stemness" genes, including ABCG2, CD133, CD24, CD34, CD44, CD117/c-kit, EpCAM, E-cadherin, β-catenin, SMO, Bmi-1, Nestin, Notch1, Notch4, Oct3/4, Oct4, and CK18. Each of genes plays an essential role in stem cell self-renewal, proliferative capacity, and fate determination [17, 18], or its corresponding protein has been used as a surface marker to identify cancer- initiating cells [4, 9, 11, 19, 20]. We found that mRNA levels of all these genes were highly expressed in all cell clones except that CD24, CD117, CD133, and E-cadherin showed a certain degree of variation between individual cell clones. The expression range of CD24, CD133, and CD117 was

from total absence to high-level. E-cadherin, required in the initial invasion stage for ovarian cancer, was also found to be expressed at a lower degree in three clones than in others (Figure 1c).

We further detected protein levels of CD24, CD117, and CD133 by flow cytometry based on their differential mRNA levels and applications in identifying CSCs in a variety of cancers. Among the clones, 0.4%-69.2% of human ovarian cancer cells expressed CD24, 0.1%-1.0% expressed CD117, and 1.2%-53.2% expressed CD133 (Figure 1d), and these three surface markers were also coexpressed in small fractions of the total population. Several representative examples of single and double marker sorted cells from a selected clone are shown (Figure 1e).

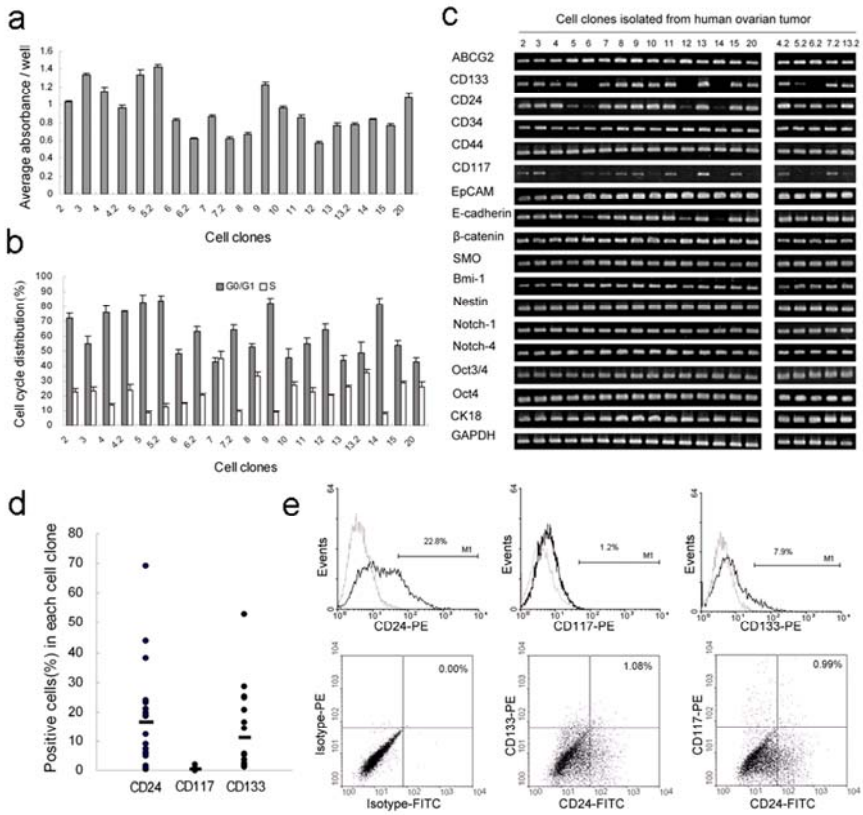

Figure 1. Isolation and characteristics of heterogeneous cell clones from human ovarian tumors. (**a**) Growth rate of 20 cell clones isolated from human ovarian tumors. Proliferation was estimated by detecting the average absorbance per well on the 5th day after cells were seeded in 96-well plates at the same density of 5000 cells/well. The experiment was carried out in quadruple and the average absorbance (±SD) of each clone indicated the growth rate. (**b**) Average cell cycle distribution in G0/G1 and S phase (±SD) of all isolated cell clones. (**c**) Expression patterns of stem cell genes in all isolated clones by RT-PCR. (**d**) Expression pattern of CD24, CD117, and CD133 in all isolated

clones by flow cytometry analyses. (**e**) Representative examples of single or double marker expression in one selected cell clone. The light-colored peak in each histogram represents the negative control and the dark color refers to the cells treated by specific antibodies.

Although CD44 mRNA levels were equal in all cell clones, we also detected its protein expression and found all 20 clonal lines varied from exhibiting 34.8% to 98.4% $CD44^+$ cells (Figure 2).

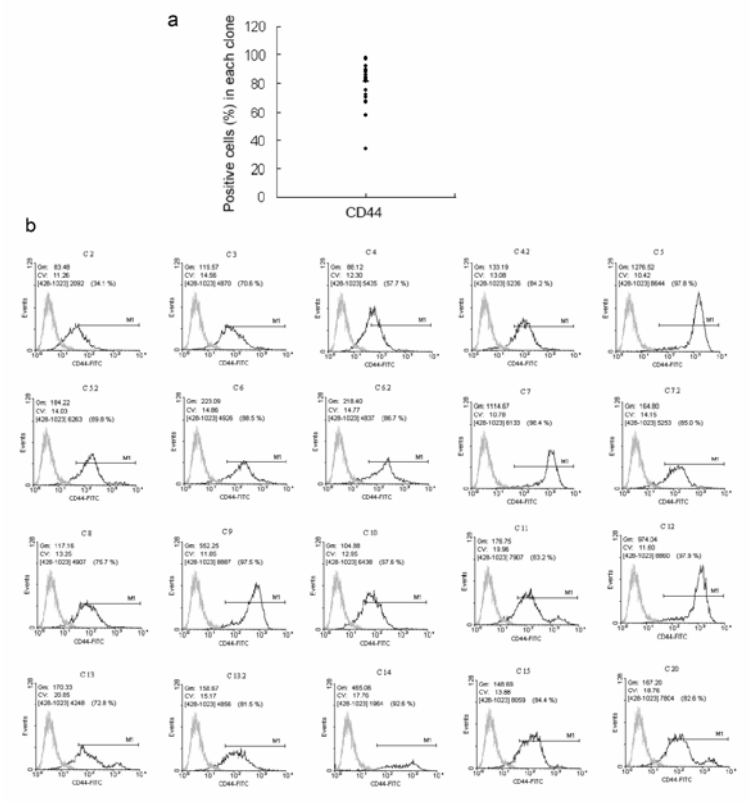

Figure 2. The expression pattern of CD44 in all isolated clones by flow cytometry analyses **(a)** and representative analyses from one of three independent experiments **(b)**.

2. Determination of potential ovarian cancer stem cell marker

The task we were faced with in the following step was to make sure which was the best choice for ovarian CSC marker in all these selected stem cell markers. In stead of doing other time-consuming and laborious studies to screen a potential ovarian CSC marker, we slightly subjectively focused on CD24, CD117, CD133, and CD44 based on the fact they were most frequently used to identify CSC in previous reports as well as their different protein expression levels in all the clonal lines. However, in view of that CD117/CD44 and CD133 have been identified as ovarian CSC markers in previous studies[13-15], we finally picked out CD24 and hypothesized it was another surface marker to identify ovarian CSCs. In addition, the correlation between expression of CD24 in ovarian tumor tissue and poor prognosis of patients [21] as well as the two-edged role of CD24 in the identification of breast and pancreatic CSCs also contributed to our final determination of CD24.

3. Growth properties of purified $CD24^+$ and $CD24^-$ cells *in vitro*

It is accepted that remaining quiescent is one of the characteristics of stem cells. To investigate which population was more quiescent between $CD24^+$ and $CD24^-$ cells, cell proliferation assay was carried out. Growth curves of sorted cells indicated that $CD24^+$ cells proliferated at a significantly lower rate than $CD24^-$ cells within 1 week after sorting ($P<0.05$, Figure 3a).

To explore the capacity of sorted cells for self-renewal and differentiation, highly purified $CD24^+$ and $CD24^-$ cells were separately cultured in vitro for 2 weeks, and then the expression of CD24 in each population was detected by flow cytometry. We found that the proportion of $CD24^+$ cells dramatically declined time dependently in purified $CD24^+$ population, and they returned to almost presorting levels; whereas $CD24^-$ tumor population was maintained in $CD24^-$ cells (Figure 3b), which indicate $CD24^+$ tumor cells arose only from $CD24^+$ population and that $CD24^-$ cell gave rise exclusively to $CD24^-$ progeny. This finding confirmed the existence of a tumor hierarchy in which $CD24^+$ ovarian cancer cells self-renewed and gave rise to more differentiated $CD24^-$ tumor progeny. We performed our analyses in triplicate, generating similar results each time.

Cisplatin is an important drug in the treatment of ovarian cancer, but one of its major limitations is that the initial response is always followed by the acquisition of drug resistance [22]. In light of the view that cancer-initiating cells play a crucial role in the development of drug-resistance[15], we examined the response of $CD24^+$ and $CD24^-$ populations to treatment with cisplatin in vitro. We determined that cisplatin IC_{50} value was greater ($P<0.01$) in the $CD24^+$ group ($IC_{50}=15.78 \pm 2.90$) than the $CD24^-$ group ($IC_{50}=8.60 \pm 2.70$). This result indicates that the $CD24^+$ fraction was relatively resistant to the chemotherapeutic agent cisplatin compared to its $CD24^-$ counterpart and may

function as CSCs in ovarian cancer chemoresistance.

Cell cycle control is one of the important aspects in CSC biology and deregulated cell cycle control is one of the fundamentally intrinsic steps leading to CSC-derived tumorigenesis [23]. However, whether CSCs relate to cell cycle distrbution is controversial, for instance, it has been suggested that neither highly tumorigenic (CSCs) nor nontumorigenic population in breast and pancreatic cancer was enriched for cells at a particular stage of the cell cycle[6,8], while human endometrial cancer lines AN3CA-derived side population cells (CSCs) accumulated in the G1 phase of the cell cycle compared to non-side population fraction [24]. To determine whether differences in proliferation, differentiation, and chemoresistance observed between $CD24^+$ and $CD24^-$ cells isolated from ovarian cancer were due to differences in the cell cycle distribution, the cell cycle status of each population were analyzed by flow cytometry. We found that $CD24^+$ population was enriched for cells at the S phase (Figure 3c) compared to $CD24^-$ cells.

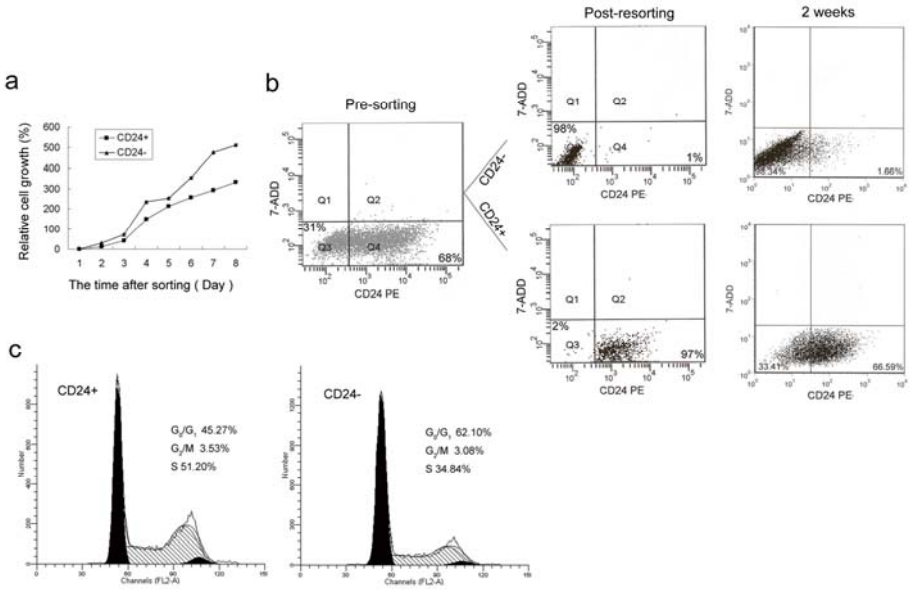

Figure 3. Characteristics of $CD24^+$ and $CD24^-$ populations in vitro. (**a**) Growth curve of $CD24^+$ and $CD24^-$ subpopulations within 8 days after sorting. Cell proliferation was detected using the XTT assay. Growth curves were prepared by the relative growth based on the percentage increase in absorbance of cells on a given day as compared to day 1, and the relative cell growth (%) = [(average absorbance in a given day/average absorbance on day 1)-1] ×100. (**b**) Differentiation of purified $CD24^+$ and $CD24^-$ cells in subculture. Dot plots represent typical examples of CD24 expression. The purity of the sorted population was >96% as revealed by post-resorting analysis. After 2 weeks in culture, the purified $CD24^+$ population generated both $CD24^+$ and $CD24^-$ cells while $CD24^-$ cells remained unchanged. (**c**) Representative histograms of cell cycle status of $CD24^+$ and $CD24^-$ cells were displayed. $CD24^+$ population was enriched for cells at S phase.

4. Enhanced aggressiveness of $CD24^+$ cells in nude mice

Heightened ability to form xenograft tumors is one of the defining characteristics of cancer-initiating cells. We therefore performed tumor xenograft experiments to investigate whether smaller numbers of $CD24^+$ cells were capable of tumorigenesis compared to $CD24^-$ cells, as previously shown for other CSCs [7, 8]. Purified $CD24^+$ and $CD24^-$ cells were injected s.c. into right and left flanks of nude mice respectively. With injection of 5×10^3 cells per mouse, $CD24^+$ cells were tumorigenic in two of three nude mice, with tumor latency of 73 and 89 days, respectively. In contrast, no tumor was observed when equal numbers of $CD24^-$ cells were injected (Figure 4a). Both the primary patient tumor and xenograft tumor were diagnosed as mucinous adenocarcinomans and exhibited similar pathological characteristics (H&E stained section; Figure 4b), with exception that the tumors in part section of xenograft looked a little worse than those of the primary tumor. In addition, the expression pattern of CD24 in the xenograft demonstrated all tumor cells were strong positive staining for CD24 (Immunohistochemistry stained section; Figure 4b).

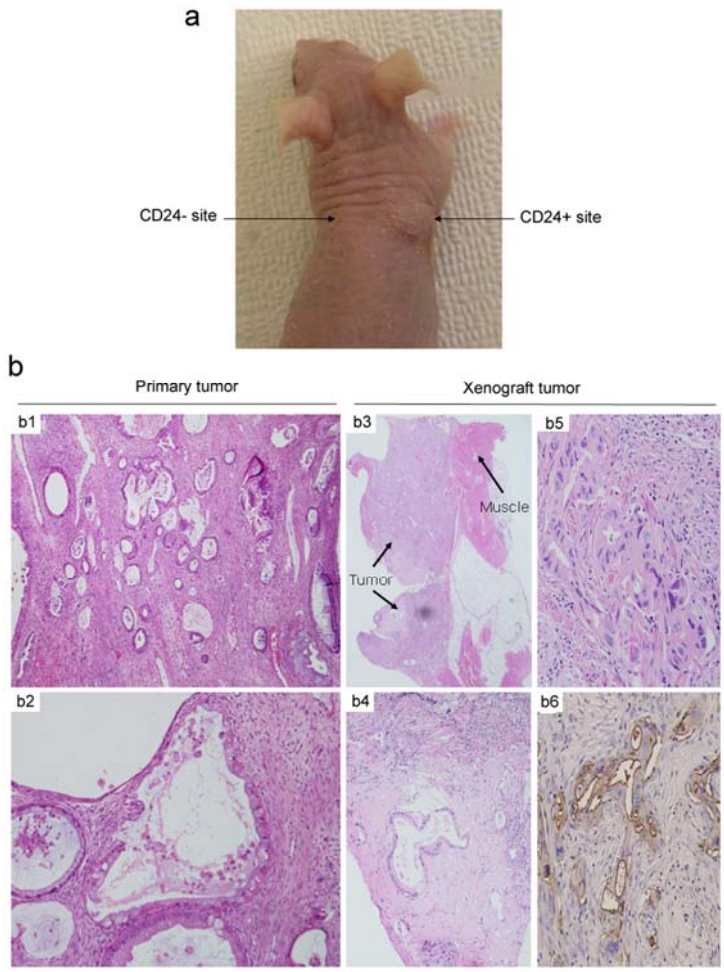

Figure 4. Tumorigenicity of $CD24^+$ and $CD24^-$ cells in nude mice. (**a**) A representative tumor in a mouse at the injection site of 5×10^3 $CD24^+$ cells, and no tumor formation at the injection site of equivalent $CD24^-$ cells. (**b**) Representative H&E staining sections of primary patient tumor and xenograft tumor. Well-formed glands with mucin were distributed (b1; 40×) and tumor cells were basally located and bland-looking (b2; 200×) in primary tumor. Identical mucinous adenocaricnoma was formed in the subcutaneous fat tissue

and back muscle of the nude mouse (b3; 10×); mucinous glands were found in the xenograft tumor mass (b4; 100×), and cytologically, xenograft tumor cells were bizarre, hyperchromatic and pleomorphic (b5; 400×). Immunohisto-chemistry staining of anti-CD24 antibody in xenograft tumor, with brown staining indicating the presence of specific antigen (b6; 400×).

5. $CD24^+$ cells preferentially expressed stem cell genes

In order to address whether $CD24^+$ cells have some other intrinsic properties conferring their stem cell-like characteristics, we examined the expression of some genes that are crucial molecules in specific signaling pathways connected with the roles of the programmes in establishing and maintaining stem cell-like characteristics. As showed in Figure 5, $CD24^+$ cells expressed higher levels of Nestin, β-catenin, Bmi-1, Oct4, Oct3/4, Notch1, and Notch4 than the matched $CD24^-$ counterpart. In contrast, similar levels of SMO and CK18 expression were observed in $CD24^+$ and $CD24^-$ cells. Increasing evidence has demonstrated that E-cadherin, a negative regulator of the canonical Wnt signaling cascade, in particular of its central mediator β-catenin, plays a crucial role in invasion and metastasis of numerous tumors [25]. Interestingly, we found $CD24^+$ cells expressed lower mRNA level of E-cadherin, with the expected association of β-catenin up-regulation, than $CD24^-$ cells, which may partly explain $CD24^+$ cells are more tumorigenic than $CD24^-$ cells in nude mice. Recently, a mechanism of

chemoresistance has been associated with the hypothesis that CSCs could efflux anticancer agents to avoid damage induced by the drug, which was mediated by ABCG2, one member of the ABC family of transporter proteins [26]. In the preceding section, we described $CD24^+$ cells were more resistant to chemotherapeutic agent than $CD24^-$ cells. Here, we also assessed the ABCG2 expression. As expected, ABCG2 was higher expressed in $CD24^+$ than $CD24^-$ population.

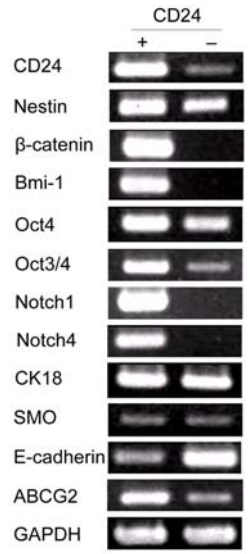

Figure 5. RT-PCR analyses of expression of stem cell genes in purified populations sorted by fluorescence-activated cell sorting against antibody of CD24. GAPDH mRNA expression was used as an internal control.

Ⅳ. DISCUSSION

The progenitor cell origin theory of cancer suggests that only a small subpopulation of cells within the tumor have stem-like properties and behave as tumor-initiating cells, which initiate not only the primary disease and but also its recurrence because of their capacity for self-renewal and inherent chemoresistance. These tumor-initiating cells, also called CSCs, are typically identified by virtue of specific cell surface markers [15, 26]. We tried to screen the potential surface markers for ovarian CSCs from lots of stem cell surface makers by using the RT-PCR, but we found it was not a good way. Because an obvious discrepancy that came up here was that the large variability in the $CD24^+$ and $CD133^+$ populations among all clonal lines didn't match the data analyzed by RT-PCR, in which 16 and 15 of 20 lines appeared to express identical amounts of CD24 and CD133, respectively. Subsequently, we further performed flow cytometry analysis to investigate the protein expression of CD44 which was equally expressed at mRNA level in all cell clones and found that all 20 clonal lines varied from exhibiting 34.8% to 98.4% $CD44^+$ cells (Figure 2), which was also unparallel to the mRNA level. This phenomenon could be explained by the saturation of amplification reaction due to excessive PCR cycles (35 cycles in our RT-PCR condition) and any variability in expression of the stem cell markers would be undetectable. Thus, it was not an effective way to screen the potential ovarian CSC markers based on the RT-PCR

analysis of selected stem cell markers. Anyway, the gene expression profiles of CD24, CD117, and CD133 analyzed by RT-PCR, as well as growth rate, cell cycle distribution, and protein expression profiles of CD24, CD117, CD133, and CD44 among all 20 clonal lines suggested that ovarian cancer cells are organized as a hierarchy.

In stead of doing other time-consuming and laborious studies to screen a potential ovarian CSC marker, we slightly subjectively focused on CD24 based on the fact it was most frequently used to identify CSC in previous reports as well as its different protein expression levels in all the clonal lines. In addition, the correlation between expression of CD24 in ovarian tumor tissue and poor prognosis of patients [21] as well as the two-edged role of CD24 in the identification of breast and pancreatic CSCs also contributed to our final determination of CD24. Here, we'd like to assert CD24 is another new-found surface marker for ovarian CSCs following the discoveries of CD133 and CD44/CD117. To date, based on stem cell behavior in normal epithelia, at least four criteria are established to indicate persistence of a stem cell pattern including (a) the capacity to remain quiescent[24], (b) generation of an amplification hierarchy[26], (c) resistance to chemotherapy[27], and (d) enhanced tumorigenicity in mice model[8]. Our experiments in vitro and in vivo suggest that $CD24^+$ cells comply to these criteria, for example, $CD24^+$ cells proliferate slowly compared to $CD24^-$ fraction and have the ability to self-renew and

differentiate into progeny ($CD24^-$) as needed; $CD24^+$ cells were more resistant to chemotherapeutic drug and possessed enhanced tumorigeniticy than the matched $CD24^-$ counterparts.

We also found that $CD24^+$ population was enriched for cells at the S phase (Figure 3c), which maybe lead to the chemotherapeutic resistance to cisplatin compared to $CD24^-$ cells, because recent work has defined cancer as a cell cycle disease, and drug therapy of malignant diseases has classically relied on cytotoxic agents, most of which act in a cell cycle specific fashion or influence the cell cycle of the malignant cells. However, the enrichment at S phase of cell cycle of $CD24^+$ cells seemed inconsistent with that $CD24^+$ cells were slower growing than $CD24^-$ cells confirmed in the preceding section, because it is generally believed that cells in the S-phase of cell cycle progression possess higher proliferation capacity than those in other cell cycle stages. The possible reason for this contradiction is that the loss of regulation that $CD24^+$ cell received within the unsorted cancer cells induced the replication of DNA in their nuclei to make a preparation for further proliferation and differentiation, nevertheless, it was insufficient for the $CD24^+$ cells to finish a cell cycle progression, thus leading to an increased S-phase but a slower cell cycle. Additionally, recent findings also revealed that cell growth was essential requirement for cell cycle progression while the growth was independent of cell cycle position. For instance, Jaksch and colleagues suggested the proliferation

rate were not significantly different between $CD133^+$ sorted Caco2 cells with DNA content of 4N or even greater (S or G2/M portion of the cell cycle) and $CD133^-$ sorted Caco2 cells with 2N DNA (G0/G1 portion of cell cycle) [28], whereas the publication by Gorannov showed that cell growth was faster in cells arrested in anaphase and G1 than in other cell cycle stages[29].

A remarkable finding in this study is that $CD24^+$ cells could form tumors in nude mice while $CD24^-$ cells have no this capability. Both the primary patient tumor and xenograft tumor were diagnosed as mucinous adenocarcinomans and exhibited similar pathological characteristics. In addition, the expression pattern of CD24 in the xenograft demonstrated all tumor cells were strong positive staining for CD24, which was different from the forenamed discovery that $CD24^+$ cells were able to differentiate into $CD24^-$ cells in vitro within 2 weeks after sorting. The possible reason is that it need take a longer time for the differentiation of $CD24^+$ cells during the tumor growth in vivo because cell differentiation is regulated by many factors, or $CD24^+$ cells produce $CD24^-$ cells only as needed, e.g., the metastasis. We should note it remains possible that the nude mice injected with $CD24^-$ fraction could develop tumors if they were monitored for a longer period. At least we can draw such a conclusion that $CD24^+$ cells were more tumorigenic than $CD24^-$ cell in nude mice.

The "stemness"gene expression profile in $CD24^+$ and $CD24^-$ cells suggest stem cell-like characteristics of $CD24^+$ cells were mediated by multiple signaling

pathways, especially those involved in the regulation of stem cell function and niche–stem cell interactions, e.g., Wnt signaling and Notch signaling. To clear which pathway plays a leading role and how to cross talk to each other, further study should be done.

It has been reported that CD24, a small glycosylphosphatidylinositol-anchored membrane protein[30], is expressed in hematological malignancies as well as in a large variety of solid tumors and is associated with an unfavorable prognosis of patients[31]. Lately, its expression in ovarian cancer has been found by chip analysis[32] and by immunohistochemistry[21], while it could not be detected in surface epithelium of normal ovaries as well as adenomas. Expression of CD24 in epithelial carcinomas of the ovary correlates with poor prognosis and is a new independent molecular marker for shortened survival time of patients with epithelial ovarian carcinomas[21]. Recent discoveries described CD24 as a potential marker for CSCs. Pancreatic CSCs expressed [8], whereas stem cells of breast carcinomas had a CD24-negative phenotype[6]. The data we described here provide a series of evidence to support that $CD24^+$ cells are ovarian CSCs contributing to tumor chemoresistance and recurrence. Therapies targeted at CD24 may provide a novel method for clinical therapeutic strategies against ovarian cancer.

Ⅴ. CONCLUSION

In conclusion, we have provided evidence that human ovarian tumor is organized as a hierarchy in growth rate, cell cycle distribution, and expression profile of genes and proteins; CD24 demarcates an ovarian cancer-initiating cell population, as indicated by the properties of relative quiescence, self-renewal and differentiation, chemoresistance, and tumorigenicity in nude mice. These findings will have important clinical applications for developing effective therapeutic strategies to treat ovarian cancer.

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ABSTRACT (in Korean)

사람 난소암의 heterogenous population으로부터 종양줄기세포의 분리 및 동정

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고명청

암줄기세포는 고형종양과 암세포주에서 확인되어 왔다. 난소암 환자의 병리검체를 분석한 결과 성장 속도, 세포 주기 분포 그리고 유전자 및 단백질의 발현이 이질적인 암세포 군락을 추출하였고, CD24 세포표면 단백질의 분포가 높은 암줄기세포의 부분 집단을 확인하였다. CD24+부분 집단은 CD24-부분에 비해서 활동이 정지되어 있는 줄기세포의 특징을 가지고 있고 화학요법에 저항성이 있으며 또한 특이적인 자가재생과 분화 능력을 가지고 있다. 게다가, CD24+ 세포 5×10^3를 누드 마우스에 주입했을 때 종양이 형성되었다. 그에 비해 같은 갯수의 CD24-세포는 종양이 형성되지 않았다. 또한 CD24+ 세포는 줄기세포 조절 유전자라고 알려진 Nestin, β-catenin, Bmi-1, Oct4, Oct3/4, Notch1 그리고 Notch4의 mRNA 수준이 높게 나타났고, 반면에 E-cadherin의 mRNA 수준은 CD24-세포에 비해 낮게 나타났다. 결과적으로 이러한 관찰들로서 난소암세포는 하나의 체계로 이루어지고 CD24 세포표면 단백질은 난소암을 유발하는

세포 집단을 구분한다는 걸 보여준다. 이러한 결과들은 난소암 치료를 하는데 있어서 효과적인 치료방법을 발전시키는 임상적인 적용을 가능하게 할 것이다.

핵심되는 말: CD24양성세포, 난소암, 암줄기세포, 종양형성

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