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Description of the CD133+ subpopulation of the human ovarian cancer cell line OVCAR3

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Abstract. Cancer stem cells (CSCs) form a very rare population within tumors and possess the ability to proliferate and self-renew indefinitely. The cluster of differentiation (CD) 133+ ovarian CSCs (OCSCs) have been identified recently and their clinical implications are about to be clarified. In this context, we use the CD133 antigen as a marker of OCSCs in OVCAR3 cells and show that microRNAs (miRNAs) are aberrantly expressed in this subpopulation. The OCSCs in the OVCAR3 cell line were identified by the monoclonal mouse anti-CD133-1 antibody (clone CD133). Microarray and real-time reverse transcription-polymerase chain reaction (RT-PCR) analyses were used to identify miRNAs with altered expression in CD133+ cells. The expression levels of dysregulated miRNAs, namely, miR-204, miR-206, miR-223, miR-9, miR-100, and miR-200c, were examined using TaqMan PCR. The RNA and protein levels of stem cell-specific genes were examined by real-time RT-PCR and Western blot analyses. Our results of microarray and real-time RT-PCR analyses revealed distinct miRNA expression profiles between CD133+ and CD133- OVCAR3 cells. The expression of stem cell-specific genes, namely, *Oct3/4*, *Sox2*, and *Nanog*, was higher in CD133+ cells than in CD133- cells while that of *FoxD3*, was lower in CD133+ cells than in CD133- cells. In conclusion, our data indicate that CD133 expression defines a tumor-initiating subpopulation of cells in the OVCAR3 cell line. The overall miRNA expression profile of CD133+ OVCAR3 cells was clearly distinct from that of CD133- OVCAR3 cells, indicating that miRNAs are involved in the development of this neoplasia and may serve as pertinent chemotherapeutic targets.

Introduction

Ovarian cancer is the fifth leading cause of cancer deaths among women and is the most common type of gynecologic malignancy. Despite advances in surgery and chemotherapy, the survival rate of patients with epithelial ovarian cancer is still low. Most ovarian cancer patients relapse and become drug-resistant after a period of medical treatment. Increasing evidence has suggested that the tumor growth potential depends on cancer stem cells (CSCs), which represent a small subset of cells within various human tumors including ovarian cancer (1-3). Cluster of differentiation (CD) 133 has been reported to be one of the main markers of ovarian CSCs (OCSCs) (1). Our study aims to further characterize CD133+ OCSCs by microarray analysis.

Materials and methods

Cell culture. The human ovarian cancer cell line OVCAR3 was seeded into T75 culture flasks at a density of $1x10^5$ cells/ml with Dulbecco's modified Eagle's medium (MEM/F-12, Gibco) supplemented with 10% fetal bovine serum, 100 μU/ml penicillin G, and 100 μg/ml streptomycin. The cells were maintained in a humidified 5% CO_2 incubator at 37˚C.

Cell sorting. The cells of interest were separated by fluorescence-activated cell sorting (FACS) under sterile conditions as described elsewhere (4). Briefly, cells to be sorted were stained with CD133/1-APC antibody (Miltenyi) (10 μl/10^7 cells) for 30 min on ice in the dark. Control cells were incubated with the culture medium without the antibody. After incubation, antibody-stained and control cells were washed once with 3 ml of ice-cold phosphate-buffered saline and centrifuged for 10 min at 4˚C at 1100 rpm. Then, 1 mg/ml propidium iodide was added and the cells were maintained on ice in the dark before FACS (EPICS, ALTRA, Beckman Coulter, Fullerton, CA, USA). Both CD133/1+ and CD133/1- cells were collected in order to evaluate the sorting efficiency and to conduct further experiments.

Isolation of low molecular weight RNA and microRNA (miRNA) microarray analysis. Total RNA was extracted using the TRIzol reagent (Invitrogen) and RNeasy Mini kit (Qiagen) according to the manufacturer's instructions. RNA was quantified using NanoDrop ND-100, followed by quality assessment with 2100 Bioanalyzer (Agilent Technologies). RNA concentrations for individual samples were >200 ng/μl, with 28S/18S ratios of >2.2. After quantification of the RNA on the NanoDrop instrument, the samples were labeled

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using the miRCURY Hy3/Hy5 Power labeling kit and were hybridized on the miRCURY LNA Array (version 11.0). Signal intensities were normalized using the global Lowess regression algorithm. To find consistently differentially expressed miRNAs, the data were subjected to significance analysis of microarrays (SAM). For visualization of differentially expressed miRNAs, a heat map was generated using the TreeView program.

RNA isolation and quantitative real-time reverse transcription-polymerase chain reaction (RT-PCR). Total RNA of sorted cells was extracted using the TRIzol reagent following the manufacturer's instructions. The miRNAs were quantified as described by the manufacturer (Applied Biosystems, Foster City, CA). Briefly, 10 ng of template RNAs were reverse transcribed using the miRCURY LNA first-strand cDNA synthesis kit and miRCURY LNA microRNA primers. Then, 1 μl of the RT product was introduced into the 25-μl PCR mixture in a 96-well plate; the PCR was performed in the Rotor-Gene 3000 thermocycler (Corbett Research) at 95˚C for 5 min, followed by 40 cycles at 95˚C for 10 sec, 60˚C for 20 sec, 72˚C for 20 sec, and 78˚C for 20 sec. The target gene expression was normalized between different samples according to the U6 RNA expression levels.

RT-PCR and real-time RT-PCR. Total RNA was extracted as previously described. Aliquots (100 ng) of total RNA were reverse transcribed using an RT kit (Takara Bio Inc., Shiga, Japan). The RT reaction was conducted using a 20-μl reaction mixture incubated at 37˚C for 15 min; the reaction was terminated by heating to 85˚C for 5 sec. For each PCR, 2 μl of the first-strand cDNA was used in the 20-μl reaction mixture (Takara Bio). The PCR consisted of an initial step at 95˚C for 30 sec; 30 cycles of 5 sec at 95˚C, 20 sec at 60˚C, and 11 sec at 72˚C; and a final elongation step at 72˚C for 10 min. The housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as a positive control. The mRNA extraction and RT-PCR experiments were repeated thrice. The PCR products were resolved by 2% agarose gel electrophoresis. The primer sequences used and the expected sizes of the PCR products, as described by Rho (5), are shown in Table I.

Real-time PCR was carried out with the Sequence Detection System (ABI Prism 7000, USA) according to the manufacturer's instructions. SYBR-Green (Takara Bio) was used for quantitative PCR as a double-stranded DNA-specific fluorescent dye. The PCR was conducted using the SYBR-Green qPCR kit (Finnzymes, Finland) with an initial denaturation for 30 sec, followed by 40 cycles at 95˚C for 5 sec and 60˚C for 31 sec. To determine the specificity of the PCR products, a melting curve was acquired by heating the product at 95˚C, cooling at 60˚C for 1 min, and then reheating slowly (0.3˚C/sec) up to 95˚C. The relative gene expression between multiple samples was quantified by normalization against endogenous GAPDH using the ΔCt method. Fold changes were calculated as $2^{-(S\Delta CT - C\Delta CT)}$. The primers used in the real-time RT-PCR assay were the same as those in the RT-PCR assay.

Protein isolation and Western blot analysis. Sorted cells were lysed in a lysis buffer containing 50 mM Tris·HCl (pH 7.4), 150 mM NaCl, and 1% Triton X-100. The proteins were separated by 7% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under denaturing conditions and were transferred to a nitrocellulose membrane. Membranes were incubated with an anti-Sox2 monoclonal antibody (1:250; Abcam, Cambridge, MA) and then with rabbit anti-mouse secondary antibody conjugated with horseradish peroxidase (1:5,000; Sigma). Immunoreactive proteins were visualized by using the enhanced chemiluminescence detection system (Amersham Pharmacia Biosciences, Piscataway, NJ).

Statistical analysis. Parametric or non-parametric analysis of variance (ANOVA) (with Bonferroni's or Dunn's multiple comparisons test) and Student's t-test were used to determine the statistical significance of data. Data were analyzed using SPSS 13.0 and are presented as the mean ± standard deviation (SD) of measurement. Statistical significance was determined by ANOVA and the significant level was defined as $p<0.05$.

Table I. Primers of stem-specific Human genes for RT-PCR.

Gene	Primer sequence	Size (bp)
GAPD	Sense GAAGGTGAAGGTCGGAGTC Antisense GAAGATGGTGATGGGATTTC	226
Oct3/4	Sense AGTGAGAGGCAACCTGGAGA Antisense CAAAAACCCTGGCACAAACT	451
Nanog	Sense CAAAGGCAAACAACCCACTT Antisense ATTGTTCCAGGTCTGGTTGC	394
SOX2	Sense AGAACCCCAAGATGCACAAC Antisense ATGTAGGTCTGCGAGCTGGT	466
FoxD3	Sense GTCGTTCAGCATCGAGAACA Antisense GGAGAGTGGCACGCTAAGAA	232

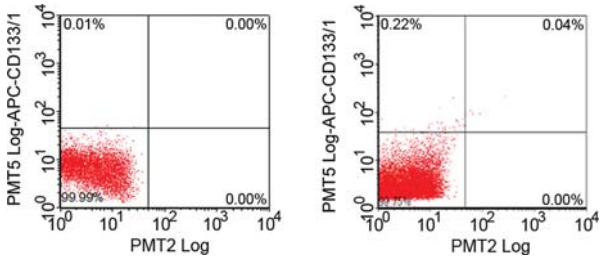

Figure 1. Cytometric analyses for CD133/1 antigen on OVCAR3 cells. CD133/1+ cell subpopulation can be detected in the OVCAR3 cells.

Results

Cell sorting. OVCAR3 cells were sorted by FACS as described above. On the basis of the fluorescence, CD133/1+ cells comprised 0.2-1% of the total cells after exclusion of dead cells and cellular debris (Fig. 1); this finding indicated that

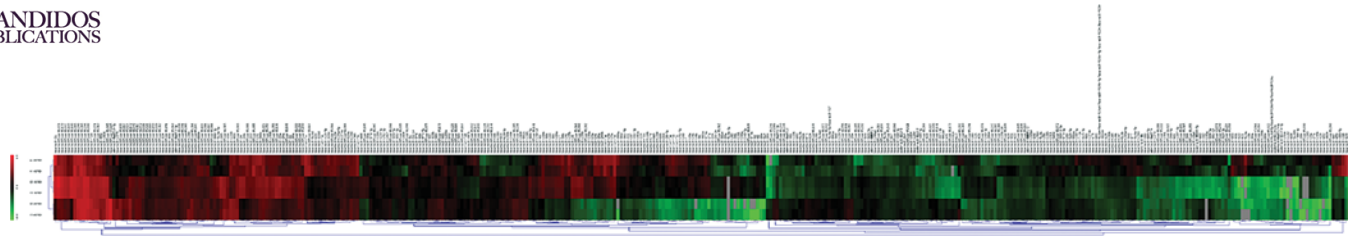

Figure 2. Heat map representation of unsupervised hierarchical clustering for the miRNA expression patterns of the CD133+ and CD133- subpopulations of the human ovarian cancer cell line OVCAR3. Vertical bars represent the samples and the horizontal bars represent the miRNAs. The heat map condition tree developed by Gene Cluster 2.0 shows microarray data of all genomic loci containing miRNAs in OVCAR3 cells. Red and green indicates gain and loss in miRNA copy number, respectively.

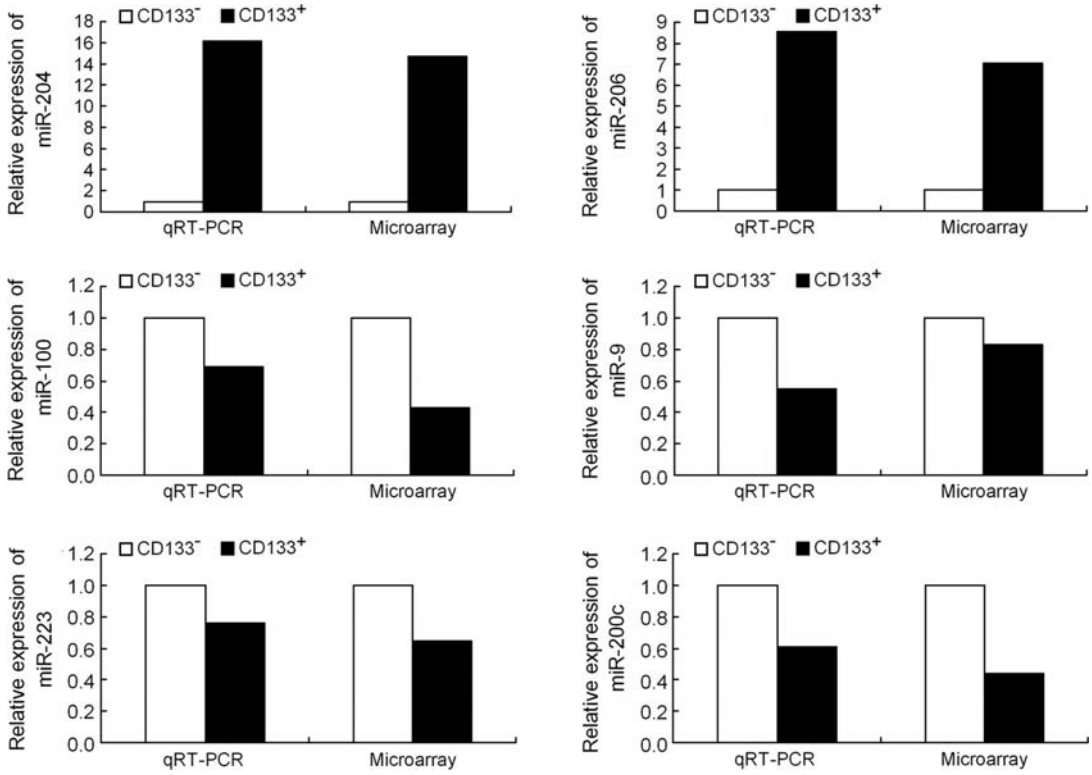

Figure 3. Validation of miRNA microarray results by quantitative RT-PCR: miRNAs differentially expressed between the two cell populations. The graphs display quantitative RT-PCR results for miRNAs differentially expressed between the cell populations; the microarray data is presented for comparison in the right segment of each graph. The expression of miR-204 and miR-206 was specifically up-regulated in CD133+ cells, while the expression of miR-200c, miR-100, miR-223, and miR-9 was strongly down-regulated in CD133+ cells (A) CD133+, black bars; (B) CD133-, white bars.

the well-known CSC marker CD133 is present at a very low ratio on the surface of OVCAR3 cells.

Distinct miRNA signatures in CD133+ ovarian cancer cells as compared to those in CD133- cells. To identify the miRNAs that were differentially expressed between CD133+ cells and CD133- cells, we used miRCURY LNA miRNA Array version 11.0, which contains 850 human miRNAs from the miRNA Registry. We analyzed the miRNA expression profiles of CD133+ and CD133- OVCAR3 cells in triplicate using a two-color system. After normalization of control oligos, the differential expression of miRNAs between the 2 subpopulations was quantified using a phosphorimager. A total of 152 miRNAs were found to be differentially expressed between CD133+ and CD133- cells (Fig. 2). The expression of 40 miRNAs was >2-fold higher and that of 112 miRNAs was >2-fold lower in CD133+ cells than in CD133- cells. Seventy-seven miRNAs were not detectable in the ovarian samples. The differential expression between the cell populations was the highest for miRPlus-C1089 (up-regulated in CD133+ cells) and mir-140-5p (down-regulated in CD133+ cells).

Validation of the microarray results. To validate the microarray results, 6 miRNAs that had >2-fold change in expression and were closely related to cancer were chosen for quantitative RT-PCR analysis (Fig. 3). In agreement with the microarray results, miR-204 and miR-206 were up-regulated, whereas miR-9, miR-100, miR-223 and miR-200c were down-regulated in CD133+ cells. Although the number of specimens is relatively small, the down-regulation of miR-9, miR-100, and miR-200c seems to be associated with CD133+ cells.

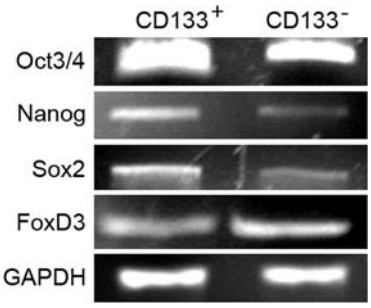

Figure 4. Expression of stem cell-specific genes in CD133+ and CD133- OVCAR3 cells by RT-PCR.

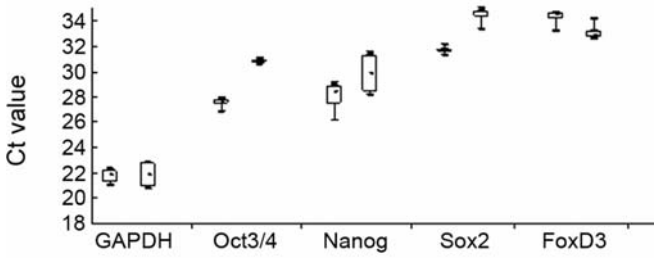

Figure 5. Expression levels of candidate endogenous control genes in CD133+ and CD133- cells. Values are given as threshold cycle (Ct) numbers. Boxes represent the lower and upper quartiles with medians; whiskers illustrate the maximum and minimum of the samples.

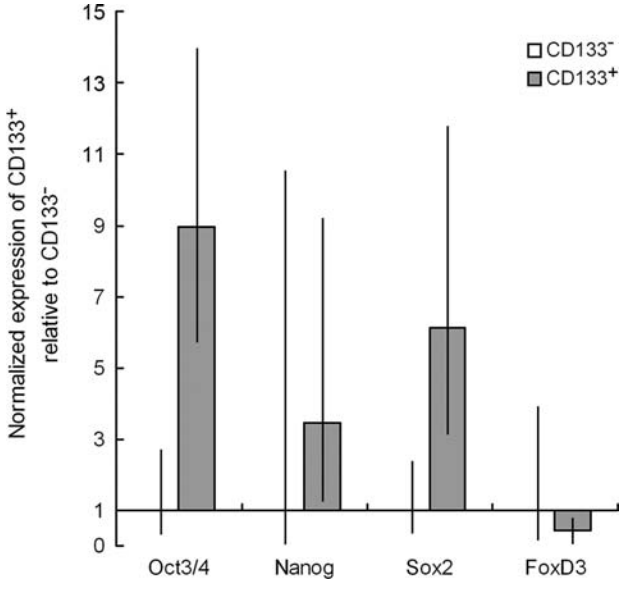

Figure 6. Normalized expression of genes between the two sample groups, relative to the calibrator (group 1, ratio = 1.0). Error bars indicate the 90% confidence interval compared with the average calibrator Ct.

Elevated expression of progenitor/stem cell genes in CD133+ cells. Expression of progenitor/stem cell genes such as *Oct-3/4*, *Sox2*, *Nanog*, and *FoxD3* was examined at the transcriptional and translational levels. RT-PCR analysis revealed that the amounts of *Oct-3/4*, *Sox2*, and *Nanog* transcripts were significantly higher in enriched CD133+ cells than in CD133- cells (Fig. 4). Real-time RT-PCR analysis further confirmed the increase in *Oct-3/4*, *Nanog*, and *Sox2* transcripts in enriched CD133+ cells (Figs. 5 and 6). In accordance with the real-time RT-PCR results, the Western blotting data showed that the Sox2 protein levels in enriched CD133+ cells were also up-regulated (Fig. 7). These results confirmed that enriched CD133+ cells possess features of CSCs, and the mRNA levels of these molecules in the signaling pathways are consistent with the protein levels.

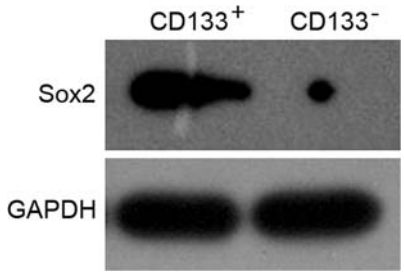

Figure 7. Western blot analysis of Sox2 protein in CD133+ and CD133- cells.

Discussion

Ovarian cancer is one of the most common malignancies among women worldwide. Despite advances in surgery and chemotherapy, the survival rate of patients is still low (6,7). Given the hypothesis that cancer is generated by a small group of cells that function in special physiological and pathophysiological settings, we compared the profiles of small RNAs between CD133+ OVCAR3 cells and CD133- OVCAR3 cells. To obtain a definitive and inclusive profile, we used an immortal cell line for profiling small RNAs. By using this approach, we avoided the effects of dissociating primary ovarian cancer tissues and changes in miRNA expression associated with the conditions used for primary cell culture.

To the best of our knowledge, this is the first report describing a complete miRNA expression profile in human OVCAR3 cells with focus on the identification of miRNAs that are differentially expressed in CD133+ cells versus CD133- cells. Using the Exiqon miRNA array, which is the most comprehensive miRNA array available, we have obtained miRNA expression signatures for the OVCAR3 cell line and have identified several miRNAs that are differentially expressed between CD133+ and CD133- cells.

We compared our results to those of other published studies that focused on the identification of miRNAs differentially expressed in carcinomas versus normal ovary (8); our data suggested that miRNAs can play an important role in the pathogenesis and development of ovarian carcinoma. One of our notable findings is that the members of the miR-200 family (miR-200a/b/c, miR-141, and miR-429) are expressed at low levels in CD133+ OVCAR3 cells and at high levels in CD133- cells. Some findings have indicated that the members of this miRNA family function in repressing the transcription factors ZEB1 and ZEB2 and thus affect the progression of epithelial-to-mesenchymal transition (9-11). Other findings suggest that the candidate targets of the miR-

ly members are stem cell factors such as Sox2 and like factor 4 (Klf4). Sox2 is an important stem cell marker co-expressed with Oct3/4 (12), and overexpression of Sox2 in patients with ovarian carcinoma usually indicates a poor prognosis (13). Klf4 is a transcription factor expressed in a wide variety of tissues in humans and is important for cellular processes such as development, differentiation, and maintenance of normal tissue homeostasis (14). Researchers have found that Klf4 expression is higher in metastatic ovarian carcinoma than in non-metastatic ovarian carcinoma (15). Moreover, miR-200c, miR-203, and miR-183 cooperate to suppress the expression of stem cell factors in cancer cells and mouse embryonic stem cells (ESCs) (16).

The GATA factors, which are involved in the mechanisms underlying dedifferentiation in ovarian carcinogenesis, have also been proposed to be targets of miR-200a and miR-200b (17). Since this miRNA family is the most highly over-expressed miRNA family in almost all ovarian cancer types, it may play a key role in ovarian carcinogenesis. Further study is needed to address this hypothesis. The miRNAs that were found to be significantly down-regulated in $CD133^+$ cells, including miR-100 (located on chromosome 11p13), are thought to be responsible for radioresistance of oral squamous cell carcinoma cells (18); further, miR-9, which was found the most down-regulated in recurrent ovarian cancers versus primary ones (19), has been reported to possess functional properties in the determination of neural fates in ES cell differentiation (20). miR-223, which was over-regulated according to Laios *et al* (19), was recently found to be myeloid-specific and negatively regulate progenitor proliferation and granulocyte differentiation and activation in mice (21).

The expression of miR-204, which was found to be up-regulated in $CD133^+$ cells, was also altered in mesenchymal progenitor cells. Further, miR-204 is predicted to bind to the 3' untranslated region of *Runx2* and down-regulate its expression; this suggests that miR-204 acts as an endogenous attenuator of *Runx2* in mesenchymal progenitor cells and bone marrow stromal cells (22). miR-206, which has been identified to function as a pro-apoptotic activator of cell death, is associated with the inhibition of notch3 signaling, tumor formation, cancer cell migration, and focus formation; these observations suggest that miR-206 may be a novel tumor suppressor (23). Our data suggested that specific miRNAs may play a role in stemgenesis through different pathways.

Curley *et al* (1) have shown that CD133 expression can define a tumor-initiating cell population in primary human ovarian tumors. Our results further suggested that $CD133^+$ cells are enriched in transcripts of stem cell-specific genes such as *Oct3/4*, *Nanog*, and *Sox2* as compared to $CD133^-$ cells; however, FoxD3, an ESC marker (24), is deregulated in $CD133^+$ OVCAR3 cells. *Oct3/4* was formerly reported to classify pluripotent cell lineages during embryo development along with *Sox2* in mice (25,26). Recently, other reports have proposed that overexpression of *Oct3/4* and *Sox2* could contribute to the pathological self-renewal characteristics of CSCs, and the overexpression is significantly associated with an unfavorable clinical outcome in human esophageal squamous cell carcinoma (15,27). Nanog, a homeodomain transcription factor, is also found to be overexpressed in more than 40 cancer types (15). Interestingly, FoxD3, which is required for the self-renewal of mouse ESCs and embryonic development in mice (28,29), was found to be down-regulated in $CD133^+$ OVCAR3 cells. This finding indicates that some signature pathways may differ between mouse and human ESCs.

Taken together, our study showed that $CD133^+$ OVCAR3 cells may display characteristics of tumor-initiating cells. Different miRNAs are expressed in $CD133^+$ and $CD133^-$ ovarian cancer cells. Since accumulating evidence has revealed the clinical impact of miRNAs, we think that further studies will provide a better understanding of miRNAs that participate in classic or novel mechanisms of activation of known pathways.

Acknowledgements

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