Ovarian cancer stem cell-specific gene expression profiling and targeted drug prescreening

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Abstract. Cancer stem cells, with unlimited self-renewal potential and other stem cell characteristics, occur in several types of cancer, including ovarian cancer (OvC). Although CSCs can cause tumor initiation, malignant proliferation, relapse and multi-drug resistance, ways to eliminate them remain unknown. In the present study, we compared ovarian cancer stem cell (OVCSC) expression profiles in normal ovarian surface epithelium and ovarian cells from patients with advanced disease to identify key pathways and specific molecular signatures involved in OVC progression and to prescreen candidate small-molecule compounds with anti-OVCSC activity. Comparison of genome-wide expression profiles of OvC stemness groups with non-stemness controls revealed 6495, 1347 and 509 differentially expressed genes in SDC, SP1 and SP2 groups, respectively, with a cut-off of fold-change set at >1.5 and P<0.05. NAB1 and NPIPL1 were commonly upregulated whereas PROS1, GREB1, KLF9 and MTUS1 were commonly downregulated in all 3 groups. Most differentially expressed genes consistently clustered with molecular functions such as protein receptor binding, kinase activity and chemo-repellent activity. These genes regulate cellular components such as centrosome, plasma membrane receptors, and basal lamina, and may participate in biological processes such as cell cycle regulation, chemoresistance and stemness induction. Key Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways such as ECM receptor, ErbB signaling, endocytosis and adherens junction pathways were enriched. Gene co-expression extrapolation screening by the Connectivity Map revealed several small-molecule compounds (such as SC-560, disulfiram, thapsigargin, esculetin and cinchonine) with potential anti-OVCSC properties targeting OVCSC signature genes. We identified several key CSC features and specific regulation networks in OVCSCs and predicted several small molecules with potential anti-OVCSC pharmacological properties, which may aid the development of OVCSC-specific drugs.

Introduction

Cancer stem cells (CSCs) have the ability to self-renew and generate heterogeneous lineages of cancer cells in a tumor. In contrast to other cell populations in a tumor, this small fraction of cells is highly tumorigenic. CSCs can survive chemotherapy and radiotherapy via efficient DNA repair and various drug-pumping mechanisms (1). Therefore, elimination of these rapidly replicating cancer cells is important to ensure successful cancer treatment. Failure of primary treatments to kill a sufficient number of CSCs can lead to relapse and metastasis, often with chemoresistance (2). In ovarian cancer (OvC), the third most common gynecological malignancy, primary cytoreductive surgery in combination with chemotherapy is initially effective. However, most patients develop drug resistance and eventually relapse within 18 months of treatment (3). Due to relapse and metastasis and the potential involvement of CSCs, the 5-year survival rate of patients with OvC continues to be <30% and their comprehensive mortality is the highest among all gynecological malignant tumors.

Ovarian cancer stem cells (OVCSCs) were first isolated and characterized after the discovery of CSCs in leukemia (4). Since then, techniques such as isolation of side population

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(SP) through flow cytometry and *in vitro* culture of ovarian multiple cellular spheroids that contain potential CSCs defined by morphology have been established to analyze the characteristics of OVCSCs. Although the Notch signaling pathway has been implicated in the development of chemoresistance in OVCSCs (5,6), small molecules targeting OVCSCs have not yet been screened. The development of such OVCSC-specific therapies may hold the key to preventing relapse and successfully treating patients who have aggressive, non-resectable OvC.

In cancer research, differentially expressed genes have been identified by comparing samples with and without CSCs by high-throughput technologies such as microarrays. Gene Ontology analysis of these differentially expressed genes may provide ontology terms to describe attributes in 3 biological domains, cellular component, molecular function and biological process, to interpret microarray data (7). The Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway query can systemically connect differentially expressed genes with other known information on the molecular interaction networks, especially in signaling pathways (8). Also, the Connectivity Map (CMAP), a massive repository of gene expression data, provides information on changes in gene expression in several cell lines when treated with >1,000 bioactive compounds (9,10). With these differentially expressed genes as query signatures of CSCs, the CMAP provides a novel resource to systematically screen for small molecules targeting CSC-specific genes.

In the present study, we used the CMAP to conduct a comprehensive analysis of multiple samples derived from the National Center for Biotechnology Information (NCBI) Gene Expression Omnibus (GEO) database to reveal several key pathways and OVCSC signature genes. Data on signature genes were then used to perform an OVCSC-specific drug prescreening based on co-expression extrapolation and predicted several small molecules with potential anti-OVCSC pharmacological properties, which may aid the development of OVCSC-specific drugs.

Materials and methods

Sources of whole-genome expression profiles of ovarian cancer. In the present study, we reanalyzed previously published raw data (Table I). Expression profiles of normal ovarian surface epithelium cells and ovarian cancer cells from patients with advanced disease were compared. The original CEL files by Rizzo *et al* (11) (GSE25191), Vathipadiekal *et al* (12)(GSE33874) and Wang *et al* (13) (GSE28799) were retrieved from the NCBI GEO database for OVCSC analysis. In brief, SPs of OvC were isolated from the serous epithelial OvC cell line IGROV1 and patient-derived ascites. Multiple cellular spheroids were isolated from the OVCAR-3 cell line on the basis of morphologic characteristics. RNA was extracted from all specimens that had OVCSC features and from non-stemness controls. Extracted RNA was pre-amplified, conjugated with fluorescent markers or biotin-labeled markers, and hybridized to an expression microarray chip. Raw data from Bonome *et al* (14) (GSE26712) were used to calibrate the OVCSC signature for drug prescreening. Serous epithelial OvC specimens were obtained independently by optimal debulking surgery from patients with previously untreated late-stage (III-IV) high-

Table I. Group designations for data derivation and input into the GeneSifter microarray analysis platform for differential expression analysis.

Name		Control	Experimental group	Series and platform	Refs.
Groups to identify stemness features	SDC	3 specimens of OVCAR-3 cells	3 specimens of OVCAR-3-derived multi-cellular spheroids	GSE28799[a]; HG-U133_Plus_2	(13)
	SP1	3 specimens of IGROV1-derived non-side population cells	3 specimens of IGROV1-derived side population cells	GSE25191[a]; HG-U133_Plus_2	(11)
	SP2	10 specimens of patient ascites-derived total ovarian cancer cells	10 specimens of patient ascites-derived side population cells	GSE33874[a]; HG-U133_Plus_2	(12)
Calibration group for drug screening	OVC	8 specimens of normal ovarian surface epithelium	8 specimens of ovarian cancer cells derived from patients with advanced disease	GSE26712[b]; HG-U133A	(14)

[a]All samples were used for analysis. [b]Eight randomly selected samples (GSM657519, GSM657520, GSM657522, GSM657524, GSM657525, GSM657526, GSM657527 and GSM657528) from 10 normal controls, and 8 samples from stage III-IV patients who received optimal postoperative assessment but due to short overall survival eventually succumbed to the disease (GSM657540, GSM 657547, GSM 657568, GSM 657575, GSM 657636, GSM 657641, GSM 657647 and GSM 657697) out of raw microarray data from 185 patients with advanced OvC.

grade (2,3) OvC. Normal ovarian surface epithelium cells were obtained by cytobrushing and RNA from these cells was independently analyzed by microarrays.

Preprocessing and normalization of array data. Four groups of raw data were normalized to probe level by using the RMA algorithm for background correction. Quantile normalization and multi-chip model mid-value fitting were performed. The final normalized data were output to the GeneSifter microarray analysis platform (http://www.geospiza.com/Products/AnalysisEdition.shtml; Geospiza, Inc., Seattle, WA, USA) by groups after logarithmic transformation.

Analysis of differentially expressed genes. In the pair wise mode, normalized microarray data were analyzed to obtain differentially expressed probes by groups and probes were then assigned to genes. The following analysis parameters were used: statistics (t-test); correction (Benjamini and Hochberg); fold-change (lower, 1.5; upper, none); and quality options (one group must pass). The Exclude Control Probes option was chosen to output both upregulated and downregulated genes with $P<0.05$.

Analysis of biological significance. Differentially expressed genes previously identified in the SDC, SP1 and SP2 groups were output to the Gene Ontology (GO) tool to determine their significance with respect to their biological processes, molecular functions and cellular components. These genes were also output to the KEGG pathway mapping tool to determine commonly enriched pathways. The Z score was used to test enrichment of GO terms and KEGG pathways in the differentially expressed gene list. A Z score of >2 or <-2 was considered statistically significant.

CMAP drug screening with cancer stem cell signature. To converge differentially expressed genes with ovarian cancer stem cell characteristics, analysis was performed by integrating upregulated and downregulated probes from each OVCSC group (SDC, SP1 and SP2) calibrated with the OvC group in the Venny tool, commonly upregulated and downregulated genes in OVCSC groups were identified in the following combinations: OVC/SDC + OVC/SP1 + OVC/SP2 + OVC/SDC/SP1 + OVC/SDC/SP2 + OVC/SP1/SP2 + OVC/SDC/SP1/SP2.

Calibration groups were introduced for drug prescreening for necessity and sufficiency:

Necessity. The CMAP database was constructed by using thousands of HG-U133A (low-density array) expression data, but in the present study all OVCSC-related signatures were derived from HG-U133_Plus_2 (high-density array). If a low-density-based filtering is not performed, the OVCSC signature will not be compatible for CMAP querying. In addition, this filtering may reduce the accuracy or significance of differentially expressed signatures for cancer stemness groups compared with that for non-stemness controls, but there were almost no intentional bias between these groups.

Sufficiency. If a calibration group with differentially expressed genes for normal ovarian surface epithelium with respect to OVCSCs containing ovarian cancer cells derived from patients with advanced disease is not introduced, the query signature will only represent massively proliferating non-stemness cancer cells over CSCs in the CAMP screening. As the small molecules with the potential of reversing the expression signature may activate CSCs by turning them into highly proliferating cancer cells, this calibration group was introduced.

CMAP drug screening by the COXEN method with the ovarian cancer stem cell signature. By using the input of OVCSC-specific query signature genes, a preliminary list of anti-OVCSC small molecules was obtained. To further screen the potential drugs targeting OVCSCs, more stringent criteria were applied by restricting the number of repeat experiments to >3 times, selecting molecules with negative enrichment score (representing the potential effectiveness), using $P<0.01$ for statistical tests, and setting the proportion of effective rate to >50%.

Results

Differentially expressed genes in OVCSC cells. By using the GeneSifter software, differentially expressed genes with a fold-change >1.5 compared with non-stemness controls and $P<0.05$ was identified for each OVCSC group. Compared with non-stemness controls, there were 6,495 (3,252 upregulated and 3,243 downregulated), 1,347 (765 upregulated and 582 downregulated) and 509 (44 upregulated and 465 downregulated) differentially expressed genes in the SDC, SP1 and SP2 groups, respectively. NAB1 (NGFI-A binding protein 1) and NPIPL1 (nuclear pore complex interacting protein-like 1) were commonly upregulated in SDC and SP1. PROS1 (protein S α), GREB1 (growth regulation by estrogen in breast cancer 1), PLCL1 (phospholipase C-like 1), MTUS1 (mitochondrial tumor suppressor 1), PPM1D (protein phosphatase 1D magnesium-dependent), CDC42EP3 (CDC42 effector protein Rho GTPase binding 3) and AMPD (adenosine monophosphate deaminase isoform E) were commonly downregulated in all OVCSC groups.

Analysis of biological significance. Enrichment analysis of differentially expressed genes revealed that 2,052, 1,005 and 1,581 biological processes were enriched in cells from SDC, SP1 and SP2 groups, respectively, as compared with non-stemness controls. Some biological processes were enriched in all OVCSC groups, such as tolerance induction, cell cycle regulation, stemness maintenance and anti-apoptosis (Table II). Distribution patterns of the involved biological processes, cellular components, and molecular functions were similar among the 3 groups (Fig. 1).

Enrichment analysis also revealed that 218, 137 and 99 cellular components were enriched in SDC, SP1 and SP2 groups, respectively. Cellular components such as membrane structures of drug resistance components, cell division components, and cell adhesion structures were enriched in all 3 groups (Table III).

In total, 597, 315 and 253 molecular functions were enriched in cells from SDC, SP1 and SP2 groups, respectively. Commonly enriched molecular functions in OVCSC cells included chemorepellent activity, growth factor receptor activity, epigenetic molecular functions and kinase activity (Table IV).

Table II. Enriched and shared biological processes from commonly upregulated or downregulated genes by 2 or 3 stemness groups.

Biological process	SDC	SP1	SP2
Enriched by upregulated genes			
Histone H3-K27 demethylation	4.12	4.31	
Histone H4-K20 demethylation	4.12	4.31	
Regulation of phosphatidylinositol 3-kinase activity	4.02	2	
Histone H3-K9 demethylation	3.64	4.09	
Negative regulation of insulin-like growth factor receptor signaling pathway	3.61	2.51	
Tolerance induction	3.04	3.56	
Negative regulation of BMP signaling pathway by extracellular sequestering of BMP	2.92	6.26	
Positive regulation of tolerance induction	2.79	2	
Regulation of tolerance induction	2.79	2	
Organ formation	2.74	2.2	
Response to growth factor stimulus	2.73	2.28	
Histone lysine demethylation	2.2	2.83	
Myoblast cell fate commitment	2.15	2.51	
Histone H3-K36 demethylation	2.15	2.51	
Positive regulation of T-cell tolerance induction	2.15	2.51	
Smooth muscle tissue development	2.04	2.7	
Bone development	2.96		2.86
Regulation of fat cell differentiation	2.88		5.73
Regulation of Wnt receptor signaling pathway	2.51		3.58
Negative regulation of response to stimulus	2.14		3.39
Regulation of canonical Wnt receptor signaling pathway	2.1		2.06
Regulation of BMP signaling pathway		3.12	3.39
Negative regulation of BMP signaling pathway		2.96	4.26
BMP signaling pathway		2.54	2.39
Interphase		2.37	2.98
Regulation of transforming growth factor β receptor signaling pathway		2.16	2.67
Interphase of mitotic cell cycle		2.11	3.03
Enriched by downregulated genes			
Embryo development	2.13	2.29	2.72
Negative regulation of transferase activity	5.37	2	2.41
Positive regulation of gene expression	2.5	2.21	3.53
Progesterone receptor signaling pathway	3.66	3.17	2.98
Regulation of toll-like receptor 3 signaling pathway	3.21	4.24	4.01
Regulation of transcription	2.12	2.21	5.16
Regulation of transcription from RNA polymerase II promoter	2.37	3.05	5.07
RNA polymerase II transcriptional preinitiation complex assembly	2.84	2.58	2.42
Epithelial cell maturation	4.14	4.48	
Endodermal digestive tract morphogenesis	2.95	7.61	
Negative regulation of tyrosine phosphorylation of STAT protein	2.19	3.17	
Hippo signaling cascade	4.64		3.27
Membranous septum morphogenesis	4.18		5.01
Tolerance induction to lipopolysaccharide	2.95		7.22
Transmembrane receptor protein tyrosine kinase signaling pathway	2.84		3.44
Cell differentiation	5.54		4.86
Canonical Wnt receptor signaling pathway	5.37		3.07
Cellular developmental process	5.37		4.64
Cell development	5.27		3.7
Regulation of Wnt receptor signaling pathway	5.26		3.02
Positive regulation of canonical Wnt receptor signaling pathway	5.22		4.52
Epithelial cell maturation involved in prostate gland development	5.12		4.01

Table II. Continued.

Biological process	SDC	SP1	SP2
Enriched by downregulated genes			
Epithelial cell differentiation involved in prostate gland development	4.83		6.2
Wnt receptor signaling pathway	4.76		3.03
Chemotaxis	4.62		2.16
Positive regulation of Wnt receptor signaling pathway	4.42		4.79
Inactivation of MAPK activity	4.24		3.73
Apoptosis in bone marrow	4.18		5.01
Regulation of apoptosis in bone marrow	4.18		5.01
Positive regulation of cell proliferation	3.99		3.52
Negative regulation of MAP kinase activity	3.96		3.09
Negative regulation of protein serine/threonine kinase activity	3.93		3.29
Cellular response to stimulus	3.7		5.21
Positive regulation of cell cycle	3.34		3.17
Cell motility	3.22		3.37
Regulation of response to stimulus	3.2		5.12
Positive regulation of response to stimulus	3.17		4.8
Apoptotic mitochondrial changes	3.16		2.67
Cell-cell adhesion	3.13		3.05
Regulation of developmental process	3.09		5.22
Positive regulation of release of cytochrome *c* from mitochondria	3.09		4.22
Epithelial cell differentiation	3.08		3.1
Release of cytochrome *c* from mitochondria	3.07		3.36
Urogenital system development	3.05		3.09
Positive regulation of mitotic cell cycle	3.05		2.33
Fibroblast growth factor receptor signaling pathway involved in positive regulation of cell proliferation	2.95		7.22
Negative regulation of CD40 signaling pathway	2.95		7.22
Negative regulation of toll-like receptor 3 signaling pathway	2.95		7.22
Regulation of CD40 signaling pathway	2.95		7.22
Regulation of release of cytochrome *c* from mitochondria	2.94		3.27
Response to stimulus	2.91		3.91
Response to external stimulus	2.87		4.43
Glandular epithelial cell differentiation	2.84		5.2
Regulation of MAP kinase activity	2.79		2.93
Response to chemical stimulus	2.73		3.41
Epithelial cell development	2.63		2.67
Fibroblast migration	2.61		3.4
Regulation of S phase	2.5		2.41
Apoptosis	2.49		6.55
Cell proliferation	2.44		4.93
Regulation of cell proliferation	2.43		5.55
Epithelium development	2.41		2.43
Programmed cell death	2.39		6.5
Regulation of cell differentiation	2.34		4.12
Cell death	2.32		5.8
Regulation of binding	2.31		4.01
Cell-cell adhesion mediated by integrin	2.28		2.04
MAPKKK cascade	2.23		3.8
Regulation of cell migration	2.21		4.07
Morphogenesis of an epithelium	2.13		3.31
Regulation of cell motility	2.13		4.02
Epithelial cell differentiation involved in mammary gland alveolus development		4.24	4.01
Negative regulation of cell growth		3.23	2.93

Table II. Continued.

Biological process	SDC	SP1	SP2
Enriched by downregulated genes			
Developmental maturation		3.23	2.2
Erythrocyte differentiation		3.16	3.89
Regulation of toll-like receptor 4 signaling pathway		2.84	5.67
Positive regulation of focal adhesion assembly		2.84	2.67
Positive regulation of macrophage differentiation		2.84	2.67
Cellular response to transforming growth factor β stimulus		2.58	2.42
Regulation of histone modification		2.44	2.44
Cell maturation		2.44	2.21
Response to transforming growth factor β stimulus		2.37	2.21
Regulation of epithelial to mesenchymal transition		2.23	3.44
Lymph vessel development		2.19	2.04
Regulation of histone methylation		2.19	4.49
Regulation of myeloid cell differentiation		2.18	3.56
Response to growth factor stimulus		2.16	2.95
Negative regulation of histone modification		2.03	4.22

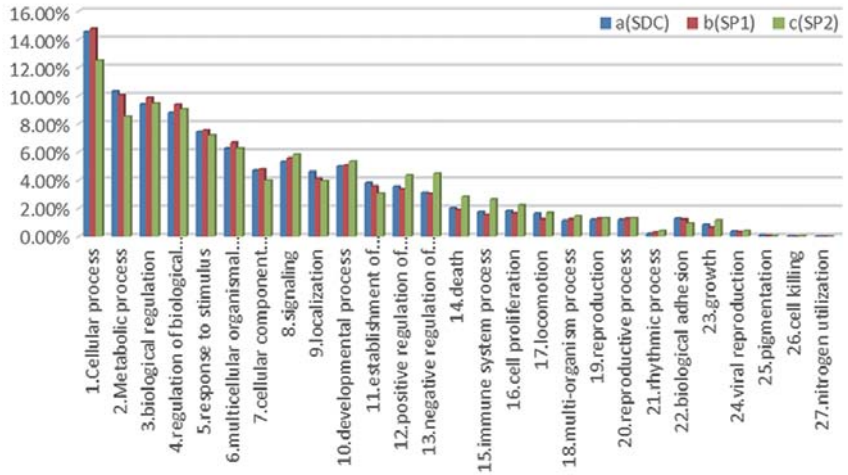

Figure 1. Distribution of the proportion of biological processes from the 3 groups of differentially expressed genes.

With regard to signaling pathways, 57, 31 and 33 KEGG pathways were enriched in cells from SDC, SP1 and SP2 groups, respectively. Key KEGG pathways such as the ErbB pathway, ECM-receptor pathway, endocytosis pathway and adherens junction pathway were enriched in all 3 groups (Table V).

Cancer stem cell signature-specific drug prescreening. The list of differentially expressed genes identified does not necessarily represent the cancerous features of OVCSCs. CSCs are highly quiescent whereas differentiated cancer cells are highly proliferative. Genes responsible for the quiescent state were also included in the above lists; however, they may not be ideal targets for therapy, as reversion of the quiescent state of OVCSCs may translate to the massive production of cancerous cells, which is undesirable. Therefore, it is necessary to focus on the cancerous features of OVCSCs. Compared with the normal ovarian surface epithelium, there were 2,669 upregulated and 3,384 downregulated genes in cells derived from patients with advanced OvC (Fig. 2). This set of genes represented cancerous features, and these genes were considered candidates for the OVCSC signature. With this calibration set, genes unrelated to the cancerous features of OVCSCs, such as those responsible for the quiescent state, were removed from the list of differentially expressed genes. Compared with non-stemness controls, there were 225 upregulated and 373 downregulated probes in the SDC group, 23 upregulated and 22 downregulated probes in the SP1 group and 5 upregulated and 38 downregulated probes in the SP2 group. There were 2 shared upregulated probes

Table III. Enriched and shared cellular components from significantly upregulated or downregulated genes by 2 or 3 stemness groups.

Cellular component	SDC	SP1	SP2
Enriched by upregulated genes			
Coated membrane	3.41	2.11	
Membrane coat	3.41	2.11	
Plasma membrane-enriched fraction	3.21	2.37	
1-Phosphatidylinositol-4-phosphate 3-kinase, class IA complex	2.96	6.57	
Basal lamina	2.77	2.75	
Polar microtubule	2.62	3.06	
Centrosome		3.15	2.33
Nuclear envelope		2.04	2.22
Enriched by downregulated genes			
Apicolateral plasma membrane	5.04	2.03	2.7
Apical junction complex	4.45	2.09	2.76
Occluding junction	4.43	2.41	3.13
Tight junction	4.43	2.41	3.13
Intracellular	4.65		2.09
Intracellular part	4.46		2.03
Nucleus	4.4		4.04
Ruffle	3.91		2.67
mRNA cap binding complex	3.4		2.06
RNA cap binding complex	3.4		2.06
Plasma membrane	2.94		2.09
Ruffle membrane	2.85		4.04
Receptor complex	2.77		3.6
Leading edge membrane	2.57		2.54
Plasma membrane part	2.45		2.02
Junctional sarcoplasmic reticulum membrane	2.21		3.01
Excitatory synapse	2.21		3.01
I-κβ/NF-κβ complex	2.21		3.01
Nuclear lumen	2.19		2.4
Basolateral plasma membrane	2.12		3.19
Neuromuscular junction	2.08		2.14
Synaptobrevin 2-SNAP-25-syntaxin-1a-complexin I complex		5.21	5.05
Synaptobrevin 2-SNAP-25-syntaxin-1a complex		4.18	4.04
Viral capsid		4.18	4.04
Virion		3.55	3.43
Virion part		3.55	3.43
Apical part of cell		2.73	2.03
Golgi lumen		2.46	2.35

and 6 shared downregulated probes between cells from SP1 and SDC groups; 7 shared downregulated probes between cells from the SDC and SP2 groups, and 1 commonly down-regulated probe between cells from the SP1 and SP2 groups compared with non-stemness controls (Fig. 3; Table VI). An OVCSC signature was generated by combining the 255 upregulated tags (23+2+225+5) and the 447 downregulated tags (22+6+373+7+38+1).

OVCSC signatures of 255 upregulated probes and 447 downregulated probes were analyzed by the CMAP for a drug prescreening. Of the 6,100 proceeded instances, it was predicted that 1,500 had the potential to promote the OVCSC signature, as indicated by their positive enrichment scores, whereas 1,419 could have anti-OVCSC effects, as reflected by their negative enrichment scores. By further filtering the 1,419 molecules with negative scores with the described criteria, 18 remained as the most promising therapeutic small-molecule candidates to target OVCSCs, for example SC-560, disulfiram (DS), thapsigargin, esculetin, cinchonine, alvespimycin and tanespimycin (Table VII).

Table IV. Enriched and shared molecular functions from significantly upregulated or downregulated genes by 2 or 3 stemness groups.

Molecular function	SDC	SP1	SP2
Enriched by upregulated genes			
Inositol 1,4,5-trisphosphate-sensitive calcium-release channel activity	5.12	3.49	
Dioxygenase activity	4.41	4.48	
Oxidoreductase activity, acting on single donors with incorporation of molecular oxygen	4.41	4.48	
Oxidoreductase activity, acting on single donors with incorporation of molecular oxygen, incorporation of 2	4.41	4.48	
Inositol-1,4,5-trisphosphate receptor activity	4.26	2.94	
Androsterone dehydrogenase activity	4.26	2.94	
trans-1,2-dihydrobenzene-1,2-diol dehydrogenase activity	4.26	2.94	
Androsterone dehydrogenase (A-specific) activity	4.18	4.38	
Glutaminase activity	4.18	4.38	
Histone demethylase activity (H3-K27 specific)	4.18	4.38	
Histone demethylase activity (H4-K20 specific)	4.18	4.38	
Oxidoreductase activity, acting on paired donors, with incorporation or reduction of molecular oxygen, 2-oxogl	3.9	3.33	
Histone demethylase activity (H3-K9 specific)	3.66	2.56	
Metal ion binding	3.57	2.09	
L-ascorbic acid binding	3.48	2.12	
Protein binding	3.13	2.66	
Calcium-dependent cysteine-type endopeptidase inhibitor activity	2.95	6.36	
Poly(G) RNA binding	2.95	6.36	
Poly-glutamine tract binding	2.95	6.36	
Chemorepellent activity	2.61	2.94	
Insulin binding	2.61	2.94	
S100 β binding	2.61	2.94	
Protein tyrosine kinase activity	2.24	3.18	
Bile acid binding	2.19	2.56	
Platelet-derived growth factor receptor binding	2.09	4.44	
Transmembrane receptor protein kinase activity	4.45		2.13
Transmembrane receptor protein tyrosine kinase activity	3.33		2.5
Virion binding		2.27	9.32
Receptor tyrosine kinase binding		2.04	4.72
Enriched by downregulated genes			
Fibroblast growth factor receptor activity	3.67	3.14	3
Protein anchor	3.22	4.21	4.03
Thiamine transmembrane transporter activity	4.19	5.25	
Thiamine uptake transmembrane transporter activity	4.19	5.25	
Uptake transmembrane transporter activity	4.19	5.25	
Reduced folate carrier activity	2.62	3.58	
General transcriptional repressor activity	2.29	4.73	
Protein serine/threonine phosphatase inhibitor activity	2.19	3.14	
Phosphatidylserine binding	2.06	2.01	
Heparan sulfate proteoglycan binding	2.06	2.01	
Interleukin-1, type II, blocking receptor activity	4.19		5.03
Epidermal growth factor receptor activity	3.22		4.03
Oncostatin-m receptor activity	3.22		4.03
Epinephrine binding	3.21		2.68
β2-adrenergic receptor activity	2.96		7.25
Calcium-dependent protein kinase c activity	2.96		7.25
Calcium-dependent protein serine/threonine kinase activity	2.96		7.25

Table IV. Continued.

Molecular function	SDC	SP1	SP2
Enriched by downregulated genes			
Cytoskeletal regulatory protein binding	2.96		7.25
Endoribonuclease activity, cleaving siRNA-paired mRNA	2.96		7.25
GTP cyclohydrolase activity	2.96		7.25
GTP cyclohydrolase i activity	2.96		7.25
Protein channel activity	2.96		7.25
Interleukin-1 binding	2.86		4
Interleukin-1 receptor activity	2.85		5.22
RNA polymerase II transcription factor binding	2.85		2.43
Sodium channel regulator activity	2.85		2.43
Actin filament binding	2.84		2.97
Protein tyrosine kinase activity	2.82		2.19
Adenylate cyclase binding	2.62		3.42
Norepinephrine binding	2.62		3.42
Insulin receptor binding	2.58		2.07
Transmembrane receptor protein tyrosine kinase activity	2.54		2.53
Co-SMAD binding	2.54		2.22
RNA cap binding	2.54		2.22
Small conjugating protein ligase activity	2.51		2.81
Map kinase tyrosine/serine/threonine phosphatase activity	2.44		3.6
Protein dimerization activity	2.41		3.03
Growth factor binding	2.4		3.47
Ubiquitin thiolesterase activity	2.37		2.68
Acid-amino acid ligase activity	2.35		2.38
Transcription activator activity	2.32		2.31
Transmembrane receptor protein kinase activity	2.31		2.78
Adrenergic receptor activity	2.29		2.05
Map kinase phosphatase activity	2.26		3.44
Ligase activity, forming carbon-nitrogen bonds	2.21		2.01
Cyclohydrolase activity	2.19		3
Prostaglandin e receptor activity	2.19		3
Protein serine/threonine kinase activator activity	2.19		3
Transforming growth factor β receptor, pathway-specific cytoplasmic mediator activity	2.19		3
Ubiquitin-protein ligase activity	2.01		3.03
A1 adenosine receptor binding		5.25	5.03
Retinoic acid binding		4.73	2.05
AMP deaminase activity		4.21	4.03
Phosphatidylinositol-4-phosphate 3-kinase activity		4.21	4.03
Adenosine receptor binding		3.14	3
N-acetylglucosamine 6-*O*-sulfotransferase activity		3.14	3
Wnt-protein binding		2.57	2.42
Thyroid hormone receptor activity		2.56	2.43
Transcription regulator activity		2.4	5
Cation:chloride symporter activity		2.35	4.84
Monovalent cation:hydrogen antiporter activity		2.35	2.22
Syntaxin binding		2.34	2.2
Transcription repressor activity		2.3	4.66
Steroid hormone receptor activity		2.29	4.2
Prostaglandin receptor activity		2.17	2.05
Ligand-dependent nuclear receptor activity		2.17	5.03

Table V. The shared KEGG pathways of differentially expressed genes in ovarian cancer stemness groups.

SDC, SP1 and SP2	SDC and SP1	SDC and SP2	SP1 and SP2
ErbB signaling pathway	Metabolic pathways	Pathways in cancer	T-cell receptor signaling pathway
Prostate cancer	Focal adhesion	Endocytosis	Amoebiasis
	ECM-receptor interaction	MAPK signaling pathway	NOD-like receptor signaling pathway
	Dilated cardiomyopathy	Adherens junction	Dorso-ventral axis formation
	Hypertrophic cardiomyopathy	Epithelial cell signaling in *Helicobacter pylori* infection	Lysine biosynthesis
	Small-cell lung cancer	B-cell-receptor signaling pathway	
	Arrhythmogenic right ventricular cardiomyopathy	Hedgehog signaling pathway	
	Peroxisome	Pathogenic *Escherichia coli* infection	
	Systemic lupus erythematosus		
	Glycosaminoglycan biosynthesis-keratan sulfate		
	Glycosylphosphatidylinositol (GPI)-anchor biosynthesis		
	D-Glutamine and D-glutamate metabolism		

Discussion

OvC has the highest mortality rates of all gynecological malignancies and the 5-year survival rates of patients with OvC remain poor (15). CSC theory can consistently illustrate many clinical and pathological features of OvC, and they have been well supported by the isolation and identification of CSCs in side populations, multi-cellular spheroids from tumor bulk (16) and OvC cell lines (4). CSCs, which often consist of a low fraction, have been established to have a general connection to OvC progression, such as relapse, migration and drug resistance. Efforts have been made to study the characteristics of CSCs in detail and to effectively eliminate them.

By comparing the genome-wide expression profiles of OVCSC-specific clinical and experimental specimens from the GEO database, we identified the differentially expressed genes shared by 3 OVCSC groups. Most of the OVCSC signature genes were closely related with respect to CSC properties and OvC progression. NGFI-A binding protein 1 has been reported to be upregulated in several OVCSC groups (17). By inhibiting the transcription factor EGR-1, NAB1 downregulates the transcription of aminolevulinic acid synthase 1 (ALAS1), leading to increase in the intracellular level of heme via feedback regulation (17). High levels of heme, together with iron-mediated oxidative stress and inflammation in patients with endometriosis, can accelerate the progression of OvC (18). In the present study, genes that were downregulated in all OVCSC groups included PROS1 (protein S α), GREB1, KLF9 (Kruppel like factor 9), MTUS1 (mitochondrial tumor suppressor 1), PPM1D (protein phosphatase 1D magnesium-dependent) and inhibitor of DNA binding 2 (ID2). The downregulation of gene PROS1, a member of the HNF4α tumor suppressor network, can promote cell proliferation and finally leads to tumor progression (19). In breast cancer, estrogen-receptor-negative cancer cells with low levels of GREB1 expression (20) have more stem cell features than other cancer cells (21). Overexpression of KLF9, a differentiation-related transcription factor, promoted differentiation of malignant glioma stem cell spheres and inhibited their proliferation *in vivo* in a xenotransplantation model (22). The downregulation of KLF9 in OVCSCs suggests that its absence may be required to maintain stemness. We also confirmed downregulation of the potential tumor suppressor gene MTUS1 in OVCSCs, which is consistent with a previous study showing that downregulation of MTUS1 and Claudin-1 (CL-1) in various human tumors is related to active proliferation, poor differentiation and poor clinical outcomes (23). PPM1D, a highly expressed oncogene in various human tumors, can inactivate CHK1 and p53 via dephosphorylation, leading to cisplatin resistance in cancer cells. Therefore, this gene is a potential target for treatment of OvC (24,25). ID2 is a new drug target, as high expression of ID2 can maintain the pluripotency of neural stem cell spheroids by direct inhibition of p53 (26). However, both PPM1D and ID2 were downregulated in the OVCSC groups, which may be due to the concealment and quiescence of CSCs, but this requires further investigation.

To identify the CSC features shared by 3 OVCSC groups, detailed Gene Ontology analysis was performed by using the GeneSifter software using domains of biological processes, cellular components and molecular functions. Differentially expressed genes were significantly enriched in biological features such as tolerance induction, cell cycle regulation, stemness maintenance and anti-apoptosis (all processes involved in OvC progression), which can easily distinguish OVCSCs from other OvC cells. By analyzing the enriched signaling pathways involving differentially expressed genes, we found that OVCSCs were different from other OvC cells in the ECM-receptor pathway, focal adhesion pathway and adherens junction

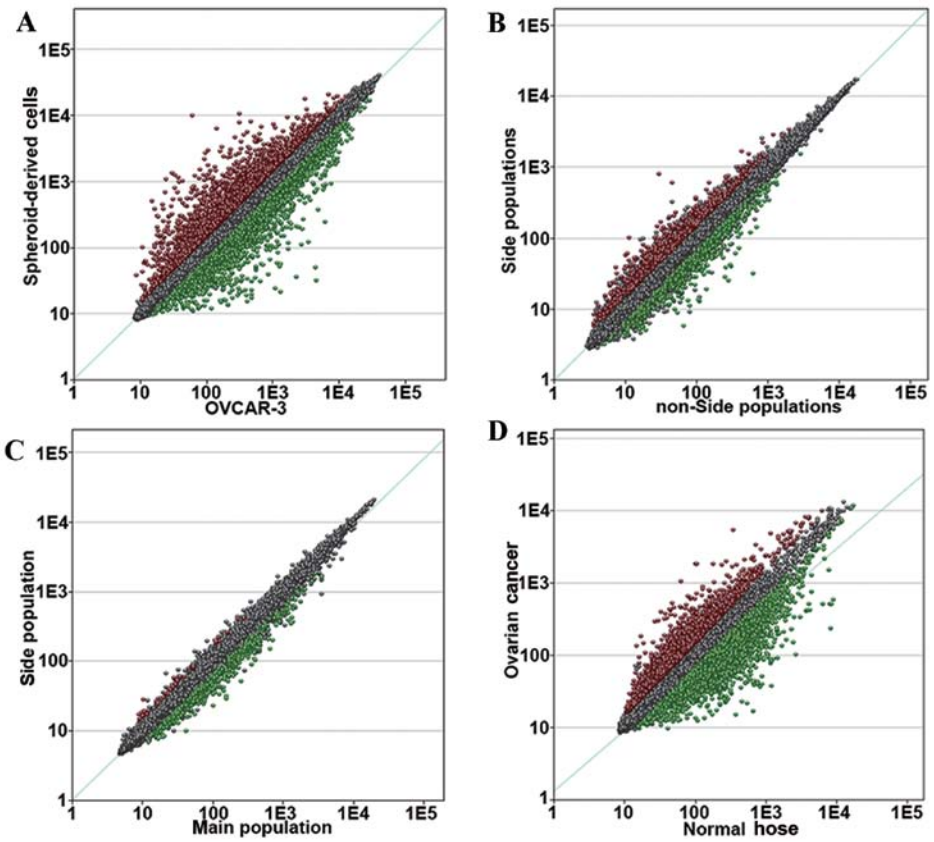

Figure 2. Scatterplot of differentially expressed genes in the (A) SDC group, (B) SP1 group, (C) SP2 group and (D) OVC group was used for drug screening calibration only. Each dot of the scatter plot represents the signal intensity of chip hybridization for every expressed gene. Those genes that neither have inconspicuous expression profiles nor match previously described criteria are represented by gray dots around or on the diagonal. Genes that have significant differential expression profiles are represented by red or green dots for upregulation or downregulation features, respectively, and are scattered far from the slash.

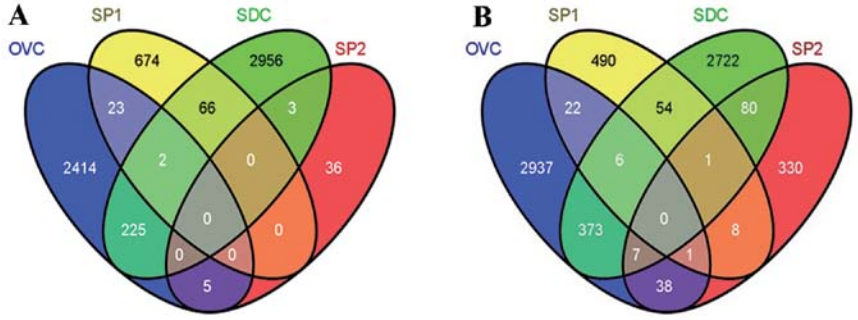

Figure 3. Shared probe numbers for (A) upregulated and (B) downregulated genes that were statistically significant among ovarian cancer stem cell groups and the calibration group.

signaling pathway. This finding is consistent with findings from other studies showing a strong correlation between these signaling pathways and epithelial stem cell proliferation, cancer invasion and migration and staged tumor progression (27,28). Moreover, some of these pathways have important functions in CSCs. The ErbB signaling pathway mediates epithelial-mesenchymal transitions in breast cancer, and is enriched in $CD44^{+}/CD24^{-}$ breast CSCs (29-31). During *Helicobacter pylori* infection, normal gastrointestinal stem cells are disrupted through epithelial cell signaling and the downstream STAT and WNT signaling pathways, leading to the genesis and progression of gastrointestinal tumors (32). These signaling pathways may also cooperate in colonic glands epithelial tumor stem cells. Pathogenic *Escherichia coli* infection correlates with the formation and progression of an epithelial colonic tumor, indicating that the same pathway is also active in OVCSCs (33). Endocytosis-related pathways are enriched in OVCSCs and represent endocytosis mediated by OVCSC-specific surface markers such as CD133 and CD44 (34).

Following characterization of signature genes in OVCSCs, co-expression extrapolation was performed with the CMAP, and small-molecule compounds with potential anti-OVCSC pharmacological properties were identified. Notably, some of these compounds [such as SC-560, disulfiram (DS), thapsigargin, esculetin, cinchonine, alvespimycin and tanespimycin] have been tested in other tumors. As a selective inhibitor of

Table VI. The probes from shared upregulated and downregulated genes in ovarian cancer stem cell groups and the calibration group.

Group	Probe	Gene name	Direction	SDC ratio (P-value)	SP1 ratio (P-value)	SP2 ratio (P-value)	OVC ratio (P-value)
SDC, SP1 and OVC	211139_s_at	NGFI-A binding protein 1 (EGR1 binding protein 1) NAB1	Up	1.6 (0.000524207987825496)	2.19 (0.00487987405631332)		1.79 (0.000889300306837179)
	215921_at	Nuclear pore complex interacting protein-like 1	Up	1.58 (0.00945304058490303)	2.09 (0.0202576342575334)		1.61 (0.0014333481542029)
	207808_s_at	Protein S (α)	Down	7.84 (0.0000735247070136605)	1.76 (0.00346916670529939)		12.32 (1.44641461217268E-08)
	205862_at	GREB1 protein	Down	3.22 (0.000988605674703398)	2.53 (0.0137390333141558)		4.36 (0.0000851847027384107)
	213931_at	Inhibitor of DNA binding 2, dominant negative helix-loop-helix protein	Down	2.85 (0.000472661276918831)	3.32 (0.00185745595436645)		3.17 (0.000972847067933643)
	203543_s_at	Kruppel-like factor 9	Down	4.98 (4.04319757064431E-07)	1.58 (0.0229481071140268)		1.92 (2.24515014734723E-06)
	201566_x_at	Inhibitor of DNA binding 2, dominant negative helix-loop-helix protein	Down	7.24 (0.0000951288294734348)	2.05 (0.0411757137373141)		1.8 (0.0095330740950358)
	205934_at	Phospholipase C-like 1	Down	1.54 (0.00586645102487323)	2.14 (0.0491165384831927)		1.55 (0.00197953042446589)
SDC, SP2 and OVC	212096_s_at	Mitochondrial tumor suppressor 1	Down	2.44 (6.10988330229413E-06)		1.68 (0.0291768466561271)	6.61 (2.61683134224164E-06)
	209288_s_at	CDC42 effector protein (Rho GTPase binding) 3	Down	2.05 (0.000033372563900896)		2.33 (0.027635397819083)	5.37 (5.86407256617617E-06)
	204224_s_at	GTP cyclohydrolase 1	Down	1.55 (0.000854582086891518)		1.84 (0.0394499654614312)	2.54 (0.0324455426569389)
	204566_at	Protein phosphatase 1D magnesium-dependent, δ isoform	Down	1.61 (0.00187102894792315)		1.72 (0.0472030826782719)	2.44 (5.76098014017778E-06)
	218182_s_at	Claudin 1	Down	4.85 (0.0000344349818731975)		1.55 (0.0482489441482889)	1.87 (0.00689245472667143)
	209286_at	CDC42 effector protein (Rho GTPase binding) 3	Down	1.9 (0.0133367282355993)		1.94 (0.0158631362361945)	1.66 (0.00733852668541466)
	205659_at	Histone deacetylase 9	Down	1.88 (9.57664107206817E-06)		2.2 (0.0335751161813233)	1.5 (0.00774593390013839)
SP1, SP2, and OVC	207992_s_at	Adenosine monophosphate deaminase (isoform E)	Down		1.94 (0.0304265496975559)	2.23 (0.0288974231347468)	2.02 (0.0000150036714772332)

Table VII. Eighteen therapeutic small-molecule drugs with potential OVCSC-specific targeting abilities in ovarian cancer.

Connectivity Map name	Mean	N	Enrichment	P-value	Specificity	% Non-null	Category
Sc-560	-0.52	3	-0.927	0.00062	0.0121	100	COX-1 inhibitor
Prestwick-1082	-0.43	3	-0.899	0.00192	0.0246	100	
Puromycin	-0.48	4	-0.865	0.00062	0.0373	100	Protein synthesis inhibitor
Doxylamine	-0.47	5	-0.854	0.00016	0.016	100	Antihistamines
Pralidoxime	-0.36	4	-0.848	0.00097	0	100	Cholinesterase reactivator
Disulfiram	-0.4	5	-0.846	0.00024	0.0072	100	Proteasome inhibitor
Thapsigargin	-0.34	3	-0.841	0.00809	0.1161	100	Non-competitive SERCA inhibitor
Esculetin	-0.32	3	-0.836	0.00877	0	100	
Phenazone	-0.39	3	-0.833	0.00931	0.0231	100	Analgesic and antipyretic
Cinchonine	-0.51	4	-0.787	0.00416	0.0617	100	Alkaloid
Lycorine	-0.29	5	-0.762	0.00144	0.08	80	Protein synthesis inhibitor
Benzthiazide	-0.17	4	-0.756	0.00716	0.0204	50	Diuretic and antihypertensive
Naltrexone	-0.22	5	-0.735	0.0027	0.0225	60	Opioid receptor antagonist
Atropine oxide	-0.21	5	-0.714	0.00417	0.0062	60	
Pyrimethamine	-0.28	5	-0.703	0.00505	0.02	80	DHFR inhibitor
Diethylstilbestrol	-0.27	6	-0.641	0.00612	0.0164	83	Synthetic nonsteroidal estrogen
Alvespimycin	-0.24	12	-0.501	0.00255	0.0797	58	Hsp90 inhibitor
Tanespimycin	-0.19	62	-0.482	0	0.0672	53	Hsp90 inhibitor

cyclooxygenase-1, SC-560 significantly inhibits cell proliferation and arrests cells in the G0/G1 phase. SC-560 can induce autophagy of colon cancer cells *in vitro* (35) and SC-560 effectively suppresses tumor growth in animals xenografted with the ovarian cancer cell line SKOV-3 by inhibiting cell proliferation and promoting apoptosis (36). Taken together, our results and those from previous studies on OVCSCs suggest that SC-560 is a promising target for OvC treatment. The presence of the recently identified CSC marker aldehyde dehydrogenase (ALDH) has been validated in many solid tumors, including breast, colon cancer and OvC (37). Disulfiram (DS), an ALDH protease inhibitor, might suppress the migration of glioma stem cells and be used as adjuvant treatment after resection and chemotherapy (38). A recent study confirmed that DS effectively inhibits the formation of breast cancer stem cell spheres while promoting the cytotoxic effect of taxol on breast cancer cell lines by simultaneously inducing reactive oxygen species and inhibiting the NF-κB signaling pathway. Thapsigargin has been tested in clinical trials for its high efficacy in targeting CSC-specific signaling pathways (39). The natural compound esculetin effectively inhibits Ras/ERK1/2-mediated *in vitro* proliferation of colon cancer cells (40). *In vivo* studies also indicate that esculetin can significantly enhance the chemotherapy effects of cisplatin by regulating the expression of p53/Akt/phosphatase while reducing side-effects such as induced nephrotoxicity and acute leucopenia (41). The alkaloid cinchonine may reverse multi-drug resistance (MDR), which is an important mechanism for chemotherapy failure. In 2001, Furusawa *et al* (42) found that cinchonine enhances doxorubicin-induced apoptosis in multi-drug-resistant P388 leukemia cells. A recent study on cervical cancer confirmed that cinchonine not only reverses the multi-drug-resistant properties of tumor cells but also has synergistic effects on taxol-induced apoptosis (43). Therefore, cinchonine may also help overcome MDR in OVCSCs. It has recently been shown that the opioid antagonist naltrexone (NTX) effectively inhibits the proliferation of OvC *in vitro* and in xenograft tumor models. NTX can significantly enhance the efficacy of cisplatin and effectively inhibit tumor progression while alleviating the cytotoxic effects of chemotherapy (44). The heat shock protein 90 (Hsp90) inhibitors alvespimycin and tanespimycin have synergistic and sensitizing effects with cisplatin and other chemotherapy drugs used to treat breast, bladder cancer, and nervous gliomas via their specific anti-CSC effects (45-47). In clinical trials, several Hsp90 inhibitors have been effective in promoting targeted therapies of OvC. The above compounds provide new options to avoiding chemotherapy failure by specifically targeting CSCs (48). In the present study, we compared gene expression profiles from several OVCSC samples with their non-stemness cancer controls. Our study revealed that OVCSCs related differentially expressed genes, enriched Gene Ontology properties and key signaling pathways, and generated an OVCSC-specific signature for screening the small-molecule compounds with potential anti-OVCSC pharmacological properties. Thus, this approach may provide new insights into developing specific drugs that target OVCSCs.

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