Molecular Medicine

Supplemental Data

Transformation of Epithelial Ovarian Cancer Stemlike Cells into Mesenchymal Lineage via EMT Results in Cellular Heterogeneity and Supports Tumor Engraftment

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SUPPLEMENTAL METHODS

1. Hoechst 33342 Staining and Side Population Sorting

Side population (SP) cell analysis can be affected by sample incubation procedure and cell viability. We found that ES-2 cells were sensitive to the disturbance of media acid-base equilibrium. After stained with Hoechst 33342 dye at 37°C for 90 min, the media which contained 3.7g/L of sodium bicarbonate ($NaHCO_3$) as recommended, became more alkaline and ~70% percent of cells died after multi-spin-resuspending procedure. The yield of ES-2 derived-SP cells was far from the demand for the next procedure and the percentage of SP cells was quite low. To better the balance of acid-base equilibrium suitable for ES-2 cells, we modify the content of staining medium by reducing the concentration of $NaHCO_3$ to 0~3g/L, resulting in more than 60% cells were gated as propidium iodide (PI)-negative viable cells. Based on cells viability and percentage of $Hoechst^{low}$ cells, we determined that 0.25g/L of $NaHCO_3$ was the ideal concentration for ES-2-SP cell enrichment.

2. Soft Agar Assay for Colony Formation

One percent of Agar was melt in a microwave and cooled to 40°C. Isovolume of 1% Agar and 2×DMEM/20% FBS was mixed thoroughly to get 0.5% Agar/1×DMEM/10%FBS at 40°C. The mixture was plated to form base agar at room temperature. The top agar was prepared as above which contained 0.35% Agarose/1×DMEM/10%FBS and was kept at 40°C. Single ES-2, HO-8910PM cells or selected SP cells and non-SP cells were resuspended in top agar media (~1,000 cells/well) and were plated onto bottom agar until gelation. The plates were incubated at 37°C in humidified incubator for 14 d. Results presented represent triplicate experiments and are expressed as mean ± SD.

3. Suspend Spheres Formation

Single SP cells or non-SP cells were placed under stem cell conditions, containing serum-free DMEM/F12, 20ng/ml recombinant human epidermal growth factor (rhEGF, R&D systems), 10ng/ml recombinant human basic fibroblast growth factor (rhFGF, R&D systems), and 0.4% bovine serum albumin (BSA, Sigma). The cell suspension was cultured in Ultra-low attachment plates (Corning) with media change twice a week. For single cell-derived spheres cultivation, sorted SP cells or non-SP cells were plated in 96 well plates by limiting dilution assay. One cell in one well was confirmed under microscope and monitored at various time points.

4. Microarray Assay

Total RNAs were extracted with TRIzol reagent (Invitrogen) from sorted-SP cells, non-SP cells, TGF-β1-induced-SP cells and human foreskin fibroblasts cell line, HS-27. The samples were labeled with Cy-3 NHS ester (GE healthcare). Fragmentation was carried out by incubating at 60°C for 30 min in fragmentation buffer (Agilent Technologies) and stopped by adding equal volume of hybridization buffer (Agilent Technologies). Fragmented target was applied to human Whole Genome Oligo Microarray (Agilent Technologies). Hybridization proceeded at 60°C for 17 h in a hybridization oven (Agilent Technologies). The hybridized array was scanned with Agilent microarray scanner. The TIFF image generated was loaded into Feature Extraction Software (Agilent Technologies) for feature data extraction. The data analysis was performed with GeneSpring 10.0. To identify the enriched genes, the data were compared to get the information of the similarity from different samples. The significantly enriched genes were selected according to the following criteria: $p \leq 0.05$ and fold change ≥2. The signal ratios of orthologous genes were used to perform clustering analysis with cluster 3.0. The sig-

nal ratio is log ratio unless specifically defined.

5. Lentiviral Transduction

Lentivirus was produced by transfection of green fluorescence protein (GFP) or red fluorescence protein (RFP) gene-driving plasmid into 293T cells and concentrated by ultracentrifuge at 4°C, 65000g for 90 min. Viral titrations were determined by infection of 293T cells with serial dilution of virus in PBS. At the day of transduction (d 0), viral particles in cell culturing medium were transferred onto HO-8910PM cells or ES-2 cells by spinocculation at 650g for 30 min. The cells were split on regular schedule. GFP-expressing or RFP-expressing cells were sorted by FACS procedure.

6. Western Blotting Analysis

Twenty micrograms of total proteins were separated by SDS-PAGE and transferred onto a PVDF membrane. The membrane was then incubated with antibodies against E-cadherin, N-cadherin and Snail1 (Cell Signal Technology) overnight at 4°C. Bounded antibodies were detected by anti-IgG conjugated with peroxidase and developed by enhanced chemiluminescence (Pierce).

7. Fluorescence Image Detection of Tumor Pathological Analysis

GFP-expressing or RFP-expressing ovarian cancer cells were injected subcutaneously into the dorsal skin of female nude mice (BALB/c-nu/nu) at the age of 4 wks. The resulting tumor was removed and frozen, sectioned by a cryostat. Fluorescence expression and staining with hematoxylin and eosin (HE) on sequential slides were detected under fluorescence microscope (Nikon).

Supplementary Table S1. Pathologic Diagnosis and Clinical Information.

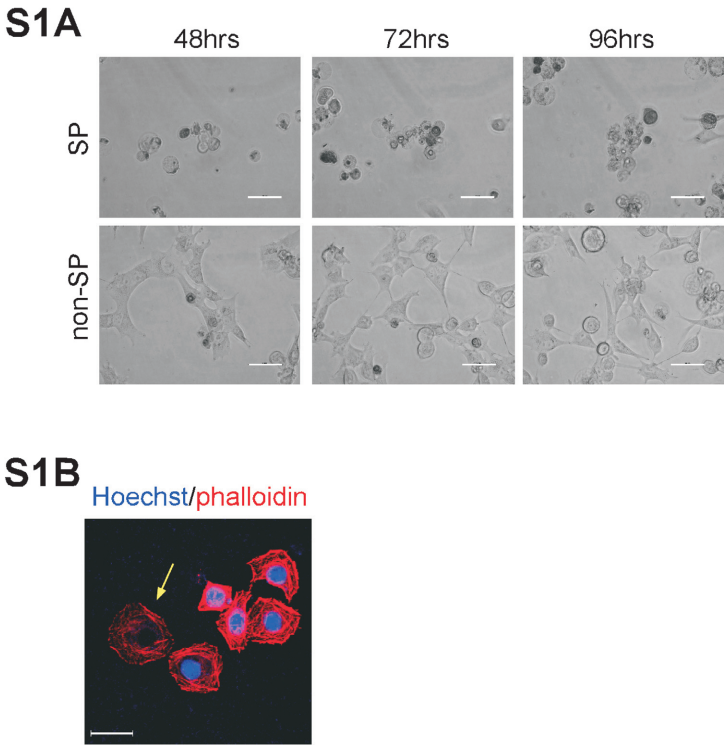
Patient	7080	7042	6014	5721
Age	40	65	73	64
Tumorigeneic organ	ovary	ovary	ovary	Fallopian tube, ovarian metastasis
Pathology	serous surface papillary borderline tumors	adenocarcinoma	adenocarcinoma, poorly differentiated	adenocarcinoma, poorly differentiated
Stage	IIIC	III	IIIC	
Primary tumor length (cm)	6	6	8	8
Volume of ascites (ml)	100	600	400	40
metastasis sites				
Peritoneum	Yes	Yes	No	No
Colon surface	Yes	Yes	Yes	No
Greater omentum	No	Yes	No	No
Uterus surface	No	No	Yes	No
Lymph node	No	Yes	No	No

Supplementary Table S2. Oligonucleotide primers used for RT-PCR analysis.

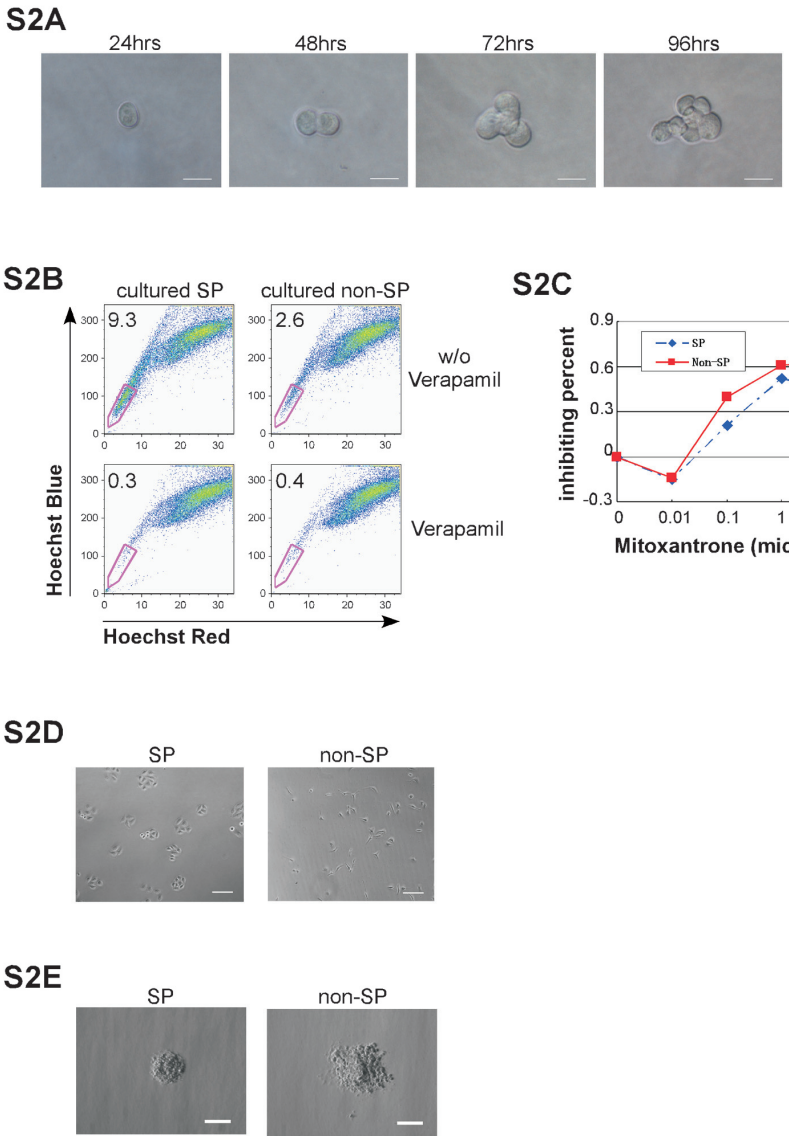
genes	5′ Primer sequence (5′-3′)	3′ Primers sequence (5′-3′)	products size (bp)
Oct-4	GACAACAATGAAAATCTTCAGGAGA	TTCTGGCGCCGGTTACAGAACCA	217
Nanog	ACTAACATGAGTGTGGATCC	TCATCTTCACACGTCTTCAG	929
BCRP	TGGCTGTCATGGCTTCAGTA	GCCACGTGATTCTTCCACAA	206
Snail1(A)	TCTGAGGCCAAGGATCTCCAG	AGCGTGTGGCTTCGGATGT	107
Snail1(B)	CCCCAATCGGAAGCCTAAC	GCCCTCCCTCCACAGAAAT	990
Snail2	GCACACTGAGTGACGCAATCA	TTGGTTGGTCAGCACAGGAGA	110
S100A4	GTCTTCCTGTCCTGCATCGC	CCCCAACCACATCAGAGGAGT	101
SIP1	AATGGCAACAGCAACAAGTGG	AACAGCCCCGTCAGCACAT	176
SMAD2	TCAGCTGCACTATGGCTGTCA	CCCCATAAGGACGCATGATTT	257
SMAD3	GCACCATCCGCATGAGCTT	TGCAAAGGCCCATTCAGGT	106
KRT8	CTGCAGTCCCAGATCTCGGA	GCGGTTGGCAATATCCTCG	117
KRT15	GAGTGAGCTCCGATGCGAGA	CGAGCAGGCTGCGGTAAGTA	107
FN1	GCACCCCACGCTCAGATACA	GGAGACAGAGGGACCCACAT	449
E-cad	TCCCTTCCCTTGAGATGA	GCCGATAGAATGAGACCCT	425
N-cad (A)	TGCTGTTTTGGACCGAGAATC	CAGCGTTCCTGTTCCACTCAT	106
N-cad (B)	TCGGGTAATCCTCCCAAAT	CCACTGCCTTCATAGTCAAA	539
Vimentin	GCCAGGCAAAGCAGGAGTC	TGGGTATCAACCAGAGGGAG	383

Supplementary Table S3. Genes Expression relating to cell adhesion in non-SP cells vs. SP cells.

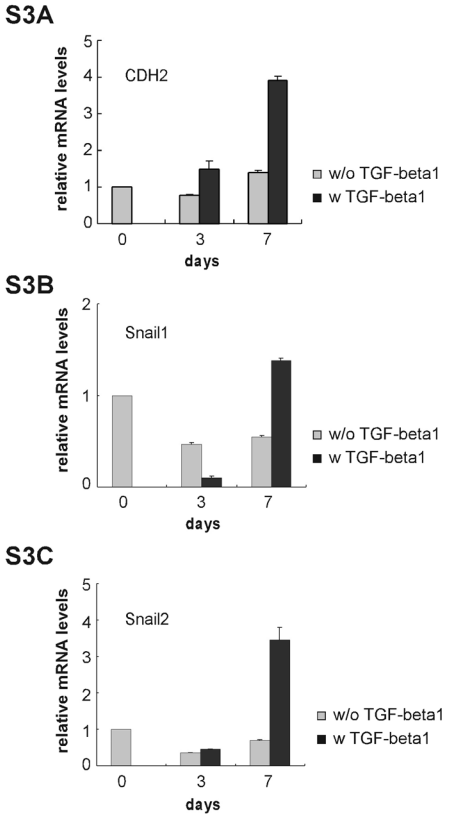
gene	definition	FC Absolute (non-SP vs. SP)	*p* value
ITGBL1	integrin, beta-like 1	9.6	0.002
PCDHGB7	protocadherin gamma subfamily B, 7	5.83	0.02
COL18A1	collagen, type XVIII, alpha 1	4.8	0.01
LAMA1	laminin, alpha 1	4.6	0.005
COL7A1	collagen, type VII, alpha 1	2.88	0.02
PCDH9	protocadherin 9	2.83	0.008
FNDC4	fibronectin type III domain containing 4	2.69	0.03
PCDHB14	protocadherin beta 14	2.46	0.03
ICAM5	intercellular adhesion molecule 5	2.29	0.04
PCDHB11	protocadherin beta 11	2.27	0.02
LY6D	lymphocyte antigen 6 complex	2.24	0.02
CLDN1	claudin 1	1.88	0.04
COL11A2	collagen, type XI, alpha 2	1.74	0.002
COL5A1	collagen, type V, alpha 1	1.71	0.001
CADM1	cell adhesion molecule 1	1.63	0.01
COL1A1	prepro-alpha1(I) collagen	1.6	0.01
LAMB2	laminin, beta 2	1.55	0.04
CLDN18	claudin 18	1.43	0.035
COL4A2	collagen, type IV, alpha 2	1.4	0.001
COL12A1	collagen, type XII, alpha 1	1.4	0.02
COL4A1	collagen, type IV, alpha 1	1.29	0.03
ITGB1	integrin, beta 1	1.28	0.05
LAMC3	laminin, gamma 3	1.25	0.015
CXCR4	chemokine (C-X-C motif) receptor 4	0.552	0.02
ITGA6	integrin, alpha 6	0.515	0.002
COL4A6	collagen, type IV, alpha 6	0.453	0.048
PCDH7	protocadherin 7	0.388	0.026
COL21A1	collagen, type XXI, alpha 1	0.328	0.004



Supplementary Figure S1. Cell Growth and F-actin expression. (A) SP cells and non-SP cells sorted from ES-2 cell line were cultured in serum-containing media for the indicated time period and detected under microscope. Scale bar = 50μm. (B) Live HO-8910PM cells were cultured adherently with Hoechst33342 and then, were fixed and co-stained with Alexa568 conjugated-phalloidin. SP cells were shown to be Hoechst33342–low cells (arrowed). Fluorescence images were detected under confocal microscope systems. Scale bar = 30μm.



Supplementary Figure S2. Characterization of ovarian cancer SP cells. (A) Sorted ES-2-SP cells were plated in 96 well plates by limiting dilution assay. One cell in one well was demonstrated under microscope and monitored at various time points. Figures were taken from the same well. Scale bar = 25μm. (B) Re-assessment of Hoechst33342-low staining cells in differentiated SP cells. Sorted ES-2-SP cells and ES-2-non-SP cells were cultured in suspended serum-free media in the presence of EGF and FGF-basic for one week and further in serum-containing media for additional one week. Trypsinized single cells were stained with Hoechst33342 and SP percentage was determined by FACS analysis (upper panels). PI stained-negative cells were gated as viable ones. Hoechst33342-low cell populations were shown to decrease in the presence of Verapamil (lower panels). Data are representative from 3 independent experiments. (C) Soft agar colony assay of SP cells and non-SP cells sorted from ES-2 cells in the presence of Mitoxantrone. After plated for 14 d, the colonies were counted under a dissecting microscope. Data are defined as the percentage of colony inhibition in the presence of Mitoxantrone compared to that in the absence of Mitoxantrone. Data are representative of three independent experiments. (D) SP cells and non-SP cells sorted from HO-8910PM cell line were cultured in serum-containing media for 48 h. Scale bar = 100μm. (E) SP cells and non-SP cells were sorted from ES-2. After plated in semisolid soft agar for 14 d, the colonies of diameter >50μm were counted under a dissecting microscope. Scale bar = 50μm.



Supplementary Figure S3. Comparison of N-Cahderin, Snail1 and Snail2 in the presence or absence of TGF-β1. Sorted HO-8910PM SP cells were cultured for 3 d and 7 d in the presence or absence of TGF-β1. Expression of CDH2 (N-Cadherin, A), Snail1 (B) and Snail2 (C) relative to unstimulated level (d 0) was determined by real-time RT-PCR.