miR-328-3p is a novel target for inhibiting cancer stem cells and preventing metastasis in ovarian cancer

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Supplemental Methods and Materials, Tables and Figures

Supplementary Methods and Materials

MicroRNA expression assay

CSCs were enriched from Kuramochi and SKOV3 cells by culturing them under CSC culture conditions as described above. RNA was isolated from these sphere cultured CSCs along with adherently cultured bulk cancer cells using Norgen Total RNA Purification Kit (Norgen Biotek, Thorold, ON, Canada) following manufacturer's instructions. miRNA expression in these samples were analyzed in the OSUCCC Nucleic Acid Core Facility using the nCounter Human v3 miRNA Expression Assay Kit (Nanostring Technologies Inc., Seattle, WA), which contains 800 human miRNAs. NanoString raw data was analyzed with nSolver™, a software provided by NanoString Technologies. Negative controls were used to perform background subtraction. Positive controls were used to perform technical normalization to adjust any lane by lane variability due to differences in hybridization, purification or binding. Data were normalized by calculating the geometric mean of the top 100 miRNAs in all samples, as recommended by NanoString. Technically normalized data was first log2 transformed (count+1 to avoid "0"s) and then a median normalization method was applied to correct for non-biological variations between different samples. Next, a filtering procedure was used to reduce number of noise level miRNAs by filtering out those miRNAs with more than 90% of the samples having expression level less than mean + 2 SD of negative controls. ANOVA was used for analysis to compare miRNA expression among groups with controlling for number of false positives among the tested miRNAs before claiming for differential expression.

Supplementary Tables

Table S1. Sequences of Primers used in this study

Genes	Forward	Reverse
Nanog	5'- GTCCCAAAGGCAAACAACCC -3'	5'- TTGACCGGGACCTTGTCTTC -3'
Sox2	5'- TCAGGAGTTGTCAAGGCAGAG -3'	5'- GGCAGCAAACTACTTTCCCC -3'
Oct4	5'- TCGCAAGCCCTCATTTCACC -3'	5'- CGAGAAGGCGAAATCCGAAG -3'
ABCG2	5'- TCATCAGCCTCGATATTCCA -3'	5'- CCTGCTTGGAAGGCTCTATG -3'
GAPDH	5'- GAAGGTGAAGGTCGGAGT -3'	5'- GAAGATGGTGATGGGATTTC -3'

Table S2. Antibodies used in this study

Antibody	Catalog number	Company
Anti-DDB2	AF3297	R&D Systems
Anti-ERK1/2	#4695	Cell Signaling Technology
Anti-phospho-ERK1/2	#9101	Cell Signaling Technology
Anti-GAPDH	Sc-365062	Santa Cruz Biotechnology
Anti-Tubulin	#2144	Cell Signaling Technology
Anti-Lamin B	#12255	Cell Signaling Technology

miRNA	**Fold Change**	
	Kuramochi	SKOV3
miR-328-3p	**199.53**	**122.94**
miR-4284	64.52	17.94
miR-630	56.77	29.3
miR-3195	28.65	5.22
miR-575	14.65	13.74
miR-4516	13.88	4.28
miR-146b-5p	12.76	4.7
miR-4488	11.98	13.75
miR-1915-3p	11.57	17.58
miR-4532	8.19	3.92
miR-320e	6.77	3.73
miR-210-3p	3.05	2.12
miR-455-3p	-16.96	-2.72
miR-379-5p	-7.16	-5.86
miR-92b-3p	-5.12	-5.16
miR-149-5p	-3.04	-2.57
miR-1260a	-2.04	-2.82

Table S3. miRNAs that up-regulated or down-regulated in CSCs compared to their corresponding bulk cancer cells in both Kuramochi and SKOV3 cell lines. The data has been deposited in the Gene Expression Ominibus (GEO GPL24289)**.**

Number	Gene Symbol	Fold Change (linear)	Number	Gene Symbol	Fold Change (linear)
1	SYNPO2	-5.53	27	PSMB9	-2.38
2	RAPGEF4	-3.89	28	PSMB9	-2.38
3	CFH	-3.88	29	FOS	-2.38
4	CFI	-3.22	30	PSMB9	-2.36
5	ARHGAP24	-3.16	31	TNFSF10	-2.35
6	PTPRZ1	-2.95	32	LOC100507376	-2.34
7	MPPED2	-2.95	33	PALMD; MIR548AA1	-2.29
8	GJA1	-2.93	34	TXNIP	-2.29
9	SUMF2	-2.88	35	PLD1	-2.26
10	STT3A	-2.84	36	BBS2	-2.25
11	DDB2	-2.80	37	PLOD2	-2.18
12	HPGD	-2.77	38	PSTPIP2	-2.16
13	PTGS2	-2.76	39	CDCA7	-2.15
14	MME	-2.73	40	MBNL3	-2.12
15	PDCD4; MIR4680	-2.70	41	MPZL2	-2.10
16	PGM2	-2.60	42	LSM14A	-2.10
17	PLCE1	-2.57	43	TAF9B	-2.09
18	NSA2	-2.57	44	SLC6A15	-2.07
19	COX6A1	-2.53	45	FRMD6	-2.05
20	PDE5A	-2.48	46	CARD16	-2.05
21	SIAE	-2.43	47	TFPI	-2.04
22	SLC1A3	-2.42	48	SUPT16H	-2.03
23	STARD7	-2.41	49	LGMN	-2.03
24	ZNF330	-2.41	50	NME1	-2.02
25	COL12A1	-2.40	51	VSNL1	-2.01
26	PSMB9	-2.39			

Table S4. Genes downregulated by overexpression of miR-328in ovarian cancer cells. OV2008 cells were transfected with miR-328-3p-M or NC miR for 3 days. Total RNA was isolated and the differentially expressed genes were screened using GeneChip Human transcriptome array 2.0. A total of 51 genes were found to be significantly downregulated with miR-328-3p overexpression (the filtering parameters are: fold change > 2, p-value < 0.01). The data has been deposited in the Gene Expression Ominibus (GEO, GSE119297).

Supplementary Figures

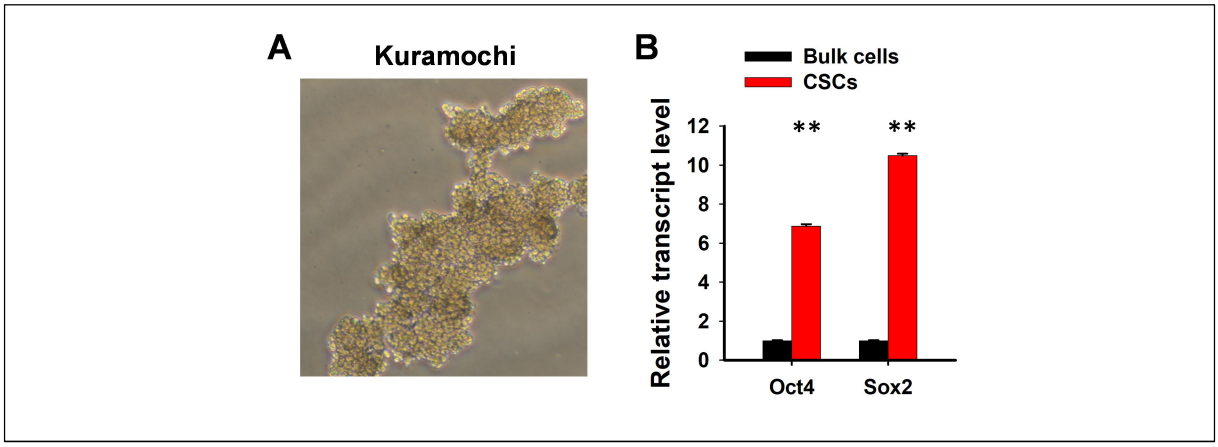


Figure S1. Sphere culture to enrich CSCs from Kuramochi cells. EOC cell line Kuramochi was cultured in CSC culture media in the Ultra-Low Attachment dishes for 12 days (A). Cells were harvested and RNA was isolated. The expression of stem cell-specific genes Oct4 and Sox2 was determined using qRT-PCR (B). N = 3, Bar: SD, **: P < 0.01 compared with bulk cells.

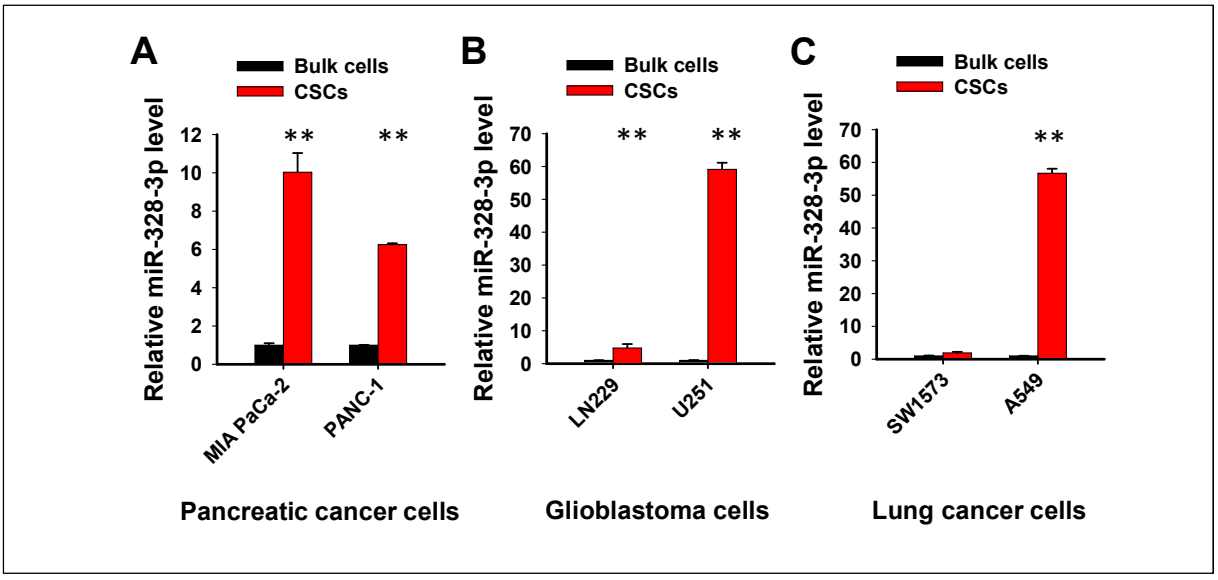


Figure S2. Enhanced miR-328-3p expression in CSCs isolated from various cancer cell lines. Pancreatic cancer cell line MIA PaCa-2 and PANC-1 (A), Glioblastoma cell line LN229 and U251 (B), and Lung cancer cell line SW1573 and A549 (C) were cultured in CSC culture media in the Ultra-Low Attachment dishes for 12 days to enrich CSCs. Cells were harvested and RNA was isolated. The expression of miR-328-3p was determined using qRT-PCR. N = 3, Bar: SD, **: P < 0.01 compared with bulk cells.

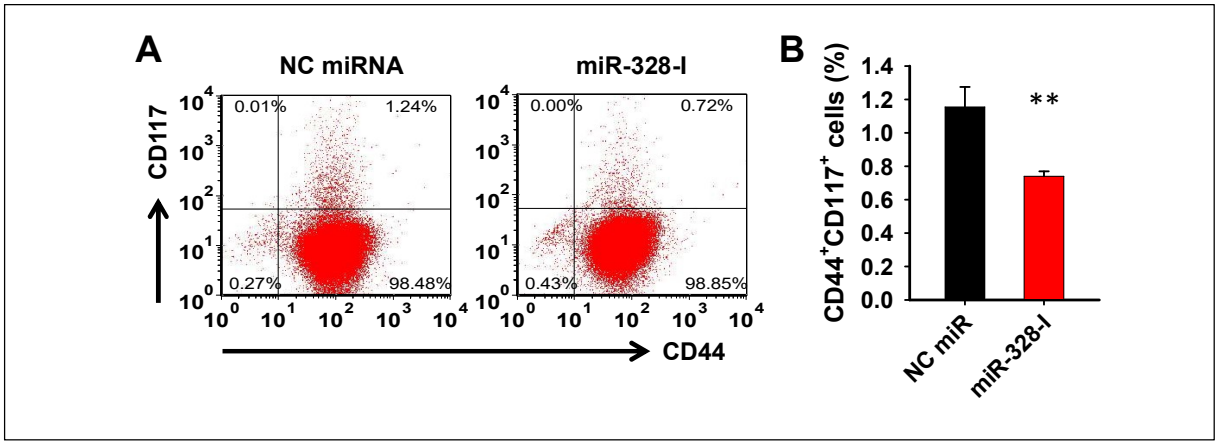
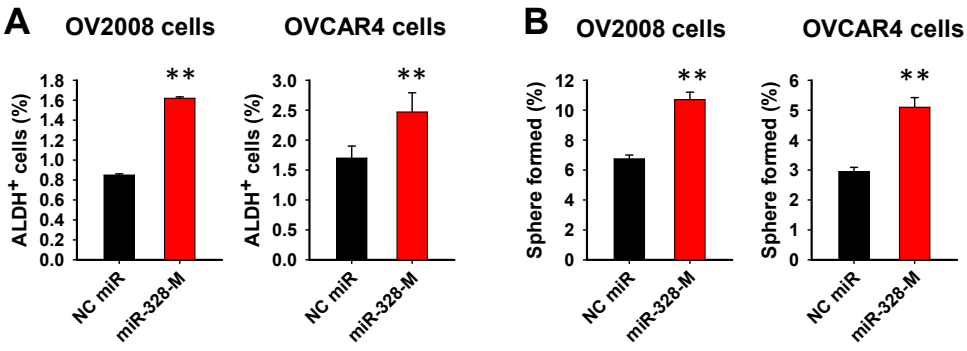


Figure S3. Inhibition of miR-328-3p reduces the CD44+CD117+ cell subpopulation in ovarian cancer cells. OV2008 cells were transiently transfected with NC miR, or miR-328-3p inhibitor (100nM) for 3 days. All cells were stained with anti-CD44-FITC and anti-CD117-PE antibodies. The percentages of CD44+CD117+ cells were analyzed using FACS (A) and plotted (B). N = 3, Bar: SD, **: P < 0.01.



C OV2008 cells

Transfection	Tumor taken after 27 days
NC miRNA	5(9)
miR-328-M	8(9)
miR-328-I	2(9)

D OVCAR4 cells

Transfection	Tumor taken after 30 days
NC miRNA	2(4)
miR-328-M	4(4)
miR-328-I	1(4)

Figure S4. miR-328 is critical to maintaining the CSC population in EOC cells. A, Overexpression of miR-328 (miR-328-M) increased the abundance of $ALDH^+$ cells in various EOC cell lines. OV2008 and OVCAR4 cells were transiently transfected with either NC miR or miR-328-M for 48 h. The proportion of $ALDH^+$ cells were analyzed with ALDEFLUOR assay using FACS. N = 3, Bar: SD, **: $P < 0.01$. **B,** Overexpression of miR-328 increased the sphere formation capability of EOC cell lines. OV2008 and OVCAR4 cells were transiently transfected with either NC miR or miR-328-M for 48 h. Semisolid colony formation assay was used to determine their sphere formation capacity. N = 3, Bar: SD, *: $P < 0.05$; **: $P < 0.01$. **C, D,** Overexpression of miR-328 enhanced while inhibition of miR-328 reduced the tumorigenic potential of the EOC cells. OV2008 and OVCAR4 cells were transfected with either NC miR, miR-328-M, or miR-328-I for 48 h. 1×10^6 cells were injected into NOD/SCID mice subcutaneously (9 mice/group for OV2008 and 4 mice/group for OVCAR4 cells. Tumor taken was observed.

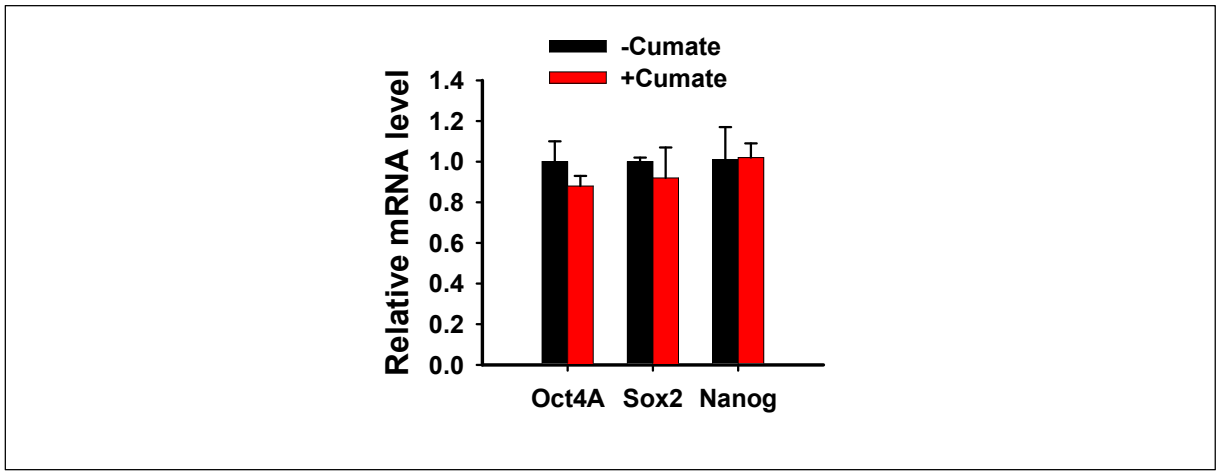


Figure S5. Cumate does not affect the stem cell-specific gene expression in ovarian CSCs. OV2008-sphere cells were treated with Cumate for 3 days, qRT-PCR was conducted to determine the expression of Sox2, Nanog, and Oct4. N = 3, Bar: SD,

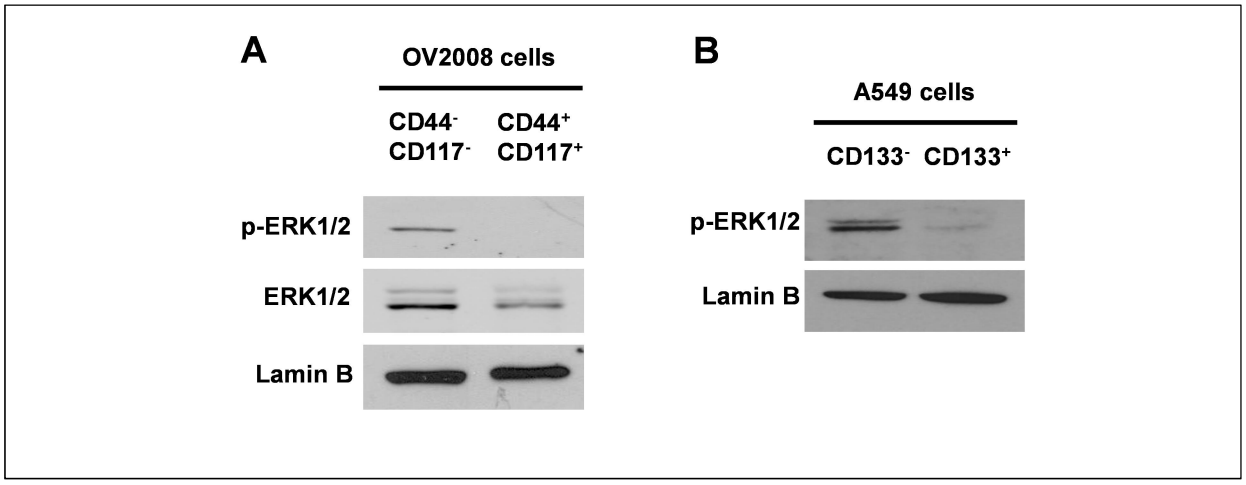


Figure S6. ERK is inactivated in CSCs isolated using surface markers. (**A)** $CD44^{-}CD117^{-}$ cells and $CD44^{+}CD117^{+}$ cells were isolated from OV2008 cells using FACS, whole cell lysates were prepared immediately after cell isolation, and phosphorylated ERK1/2 (p-ERK1/2) was determined using Western blotting. (**B)** $CD133^{-}$ and $CD133^{+}$ cells were isolated from NSCLC cell line A549 using FACS, whole cell lysates were prepared and p-ERK was determined using Western blotting.

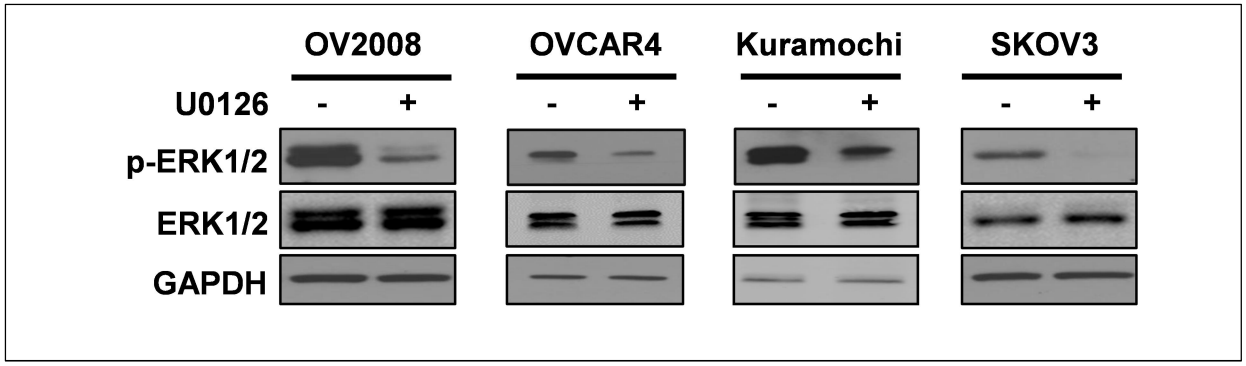


Figure S7. ERK is inhibited by U0126 in various ovarian cancer cell lines. A panel of ovarian cancer cell lines was treated with the ERK inhibitor U0126 for 24 h. Whole cell lysates were prepared and subjected to immunoblotting to determine the expression level of p-ERK1/2.

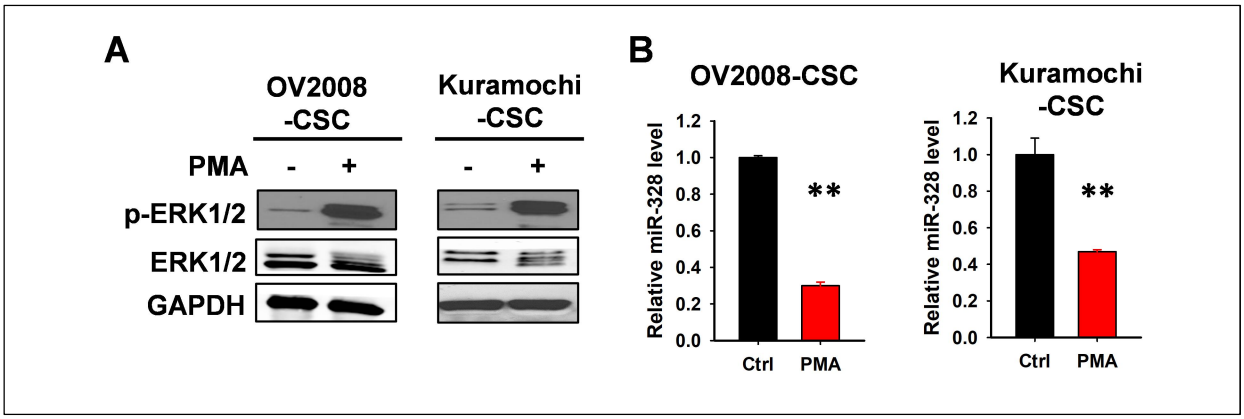


Figure S8. Activation of ERK signaling with PMA reduced miR-328-3p expression. CSCs were enriched from OV2008 and Kuramochi cells by sphere culture. Cells were treated with PMA for 24 h, the p-ERK1/2 expression level was determined using immunoblotting (A); miR-328-3p expression level was determined using qRT-PCR (B). N = 3, Bar: SD, **: P < 0.01.

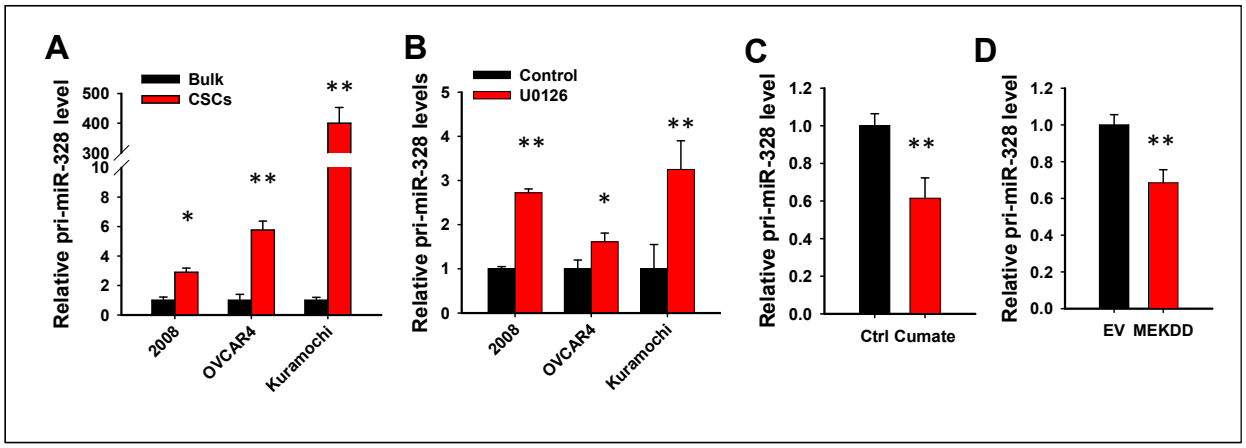
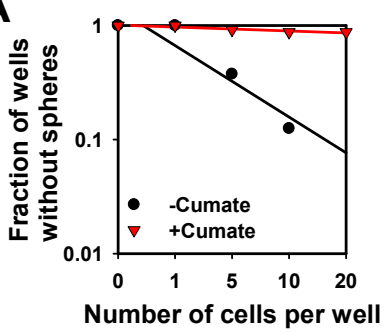


Figure S9. ERK signaling regulates cellular miR-328 levels by influencing pri-miR-328 transcription. (**A**) EOC cell line OV2008, OVCAR4, and Kuramochi cells were cultured either as monolayer in RPMI-1640 medium supplemented with 10% FBS (Bulk) or as spheres in CSC medium in ultra-low attachment dishes (CSCs). RNA was isolated and the pri-miR-328 expression level was determined using the TaqMan Pri-miRNA Assay. (**B**) EOC cell line OV2008, OVCAR4, and Kuramochi cells cultured as monolayer in RPMI-1640 medium supplemented with 10% FBS were treated with U0126 for 24 h. RNA was isolated and the pri-miR-328 expression level was determined using the TaqMan Pri-miRNA Assay. (**C**) 2008-SparQ-MEKDD cells were cultures as spheres, treated with cumate for 3 days to induce MEKDD (constitutively activated MEK1) expression. RNA was isolated and the pri-miR-328 expression level was determined using the TaqMan Pri-miRNA Assay. (D) OVCAR4 cells were cultured as sphere, transfected with either empty vector (EV) or MEKDD expression vector for 2 days. RNA was isolated and the pri-miR-328 expression level was determined using the TaqMan Pri-miRNA Assay. N = 3, Bar: SD, **: P < 0.01.

A



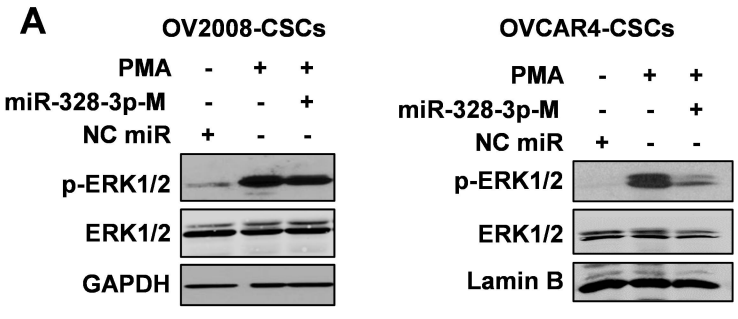
SFCf

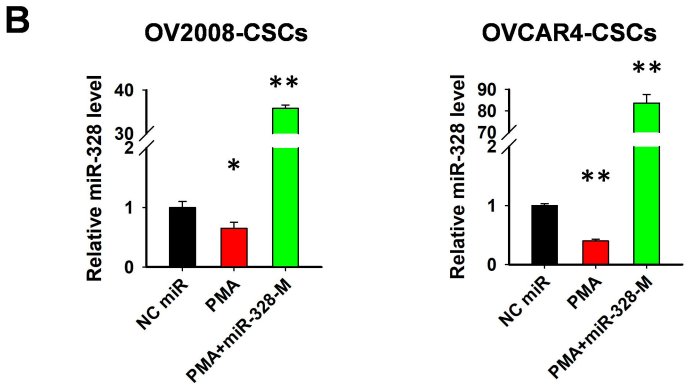
Group	Estimate (Range)	P
-Cumate	1 in 7.0 (5.2-9.4)	0.000
+Cumate	1 in 111 (53.2-234)	

B

Cell number	No. of Tumor (injection sites)	
	Control	Cumate
1×10^5	6(6)	5(6)
1×10^4	5(6)	4(6)
1×10^3	3(6)	0(6)
TICf	1/3,656	1/31,040
TICf (TIC/10^6)	273.4	32.2
P	0.000352	

Figure S10. Activation of the ERK signaling inhibits CSC properties. (**A**) Activation of the ERK signaling by cumate treatment of 2008-SparQ-MEKDD CSCs compromised their sphere formation capability. 2008-SparQ-MEKDD CSCs were treated with or without cumate for 3 days. Cells were plated in a 96-well ultra-low attachment plate in a limiting dilution manner, and the number of wells containing spheres was calculated after 10 days to calculate the SFCf. (**B**) Activation of the ERK signaling compromised the tumorigenic potential of ovarian CSCs. 2008-SparQ-MEKDD CSCs were treated with or without cumate for 3 days. The xenotransplantation limiting dilution assay was used to estimate the TICf in these cells.





C

OV2008-CSCs

Treatment	No. of Tumor /injection sites
Control	3/4
PMA	1/4
PMA+ miR-328-M	3/4

OVCAR4-CSCs

Treatment	No. of Tumor /injection sites
Control	5/6
PMA	1/6
PMA+ miR-328-M	4/6

Figure S11. Activation of the ERK signaling compromises the CSC properties through downregulation of miR-328-3p. OV2008-CSCs (spheres) and OVCAR4-CSCs (spheres) were treated with PMA or/and transfected with miR-328-3p-M for 48 h. p-ERK1/2 was detected using Western blotting (A), miR-328 expression was determined using qRT-PCR (B). N = 3, Bar: SD, *: $P < 0.05$, **: $P < 0.01$. The tumorigenic potential was determined using the xenograft assay (C). 1 X 10^5 cells were injected into NOD/SCID mice subcutaneously (4 injection sites/group in OV2008-CSCs, and 6 injection sites/group in OVCAR4-CSCs). The number of xenografts was counted after 20 and 30 days, respectively.

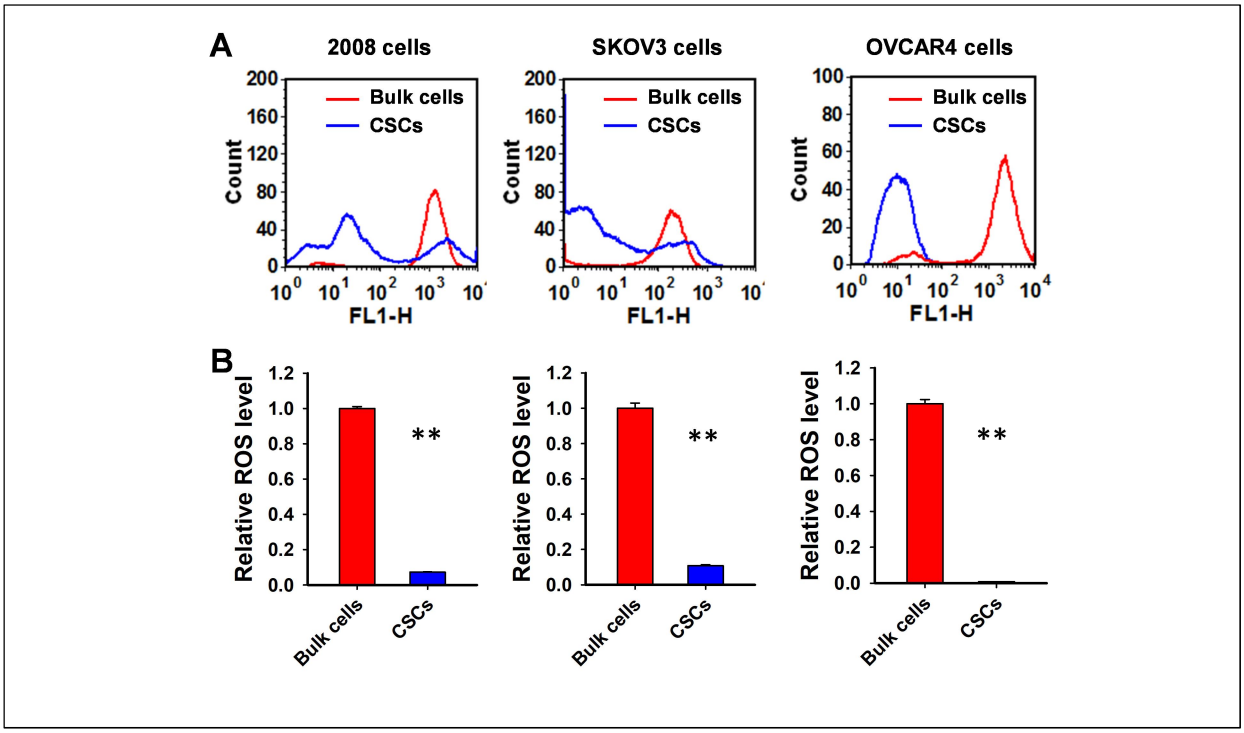


Figure S12. Ovarian CSCs possess low level of ROS. EOC cell line OV2008, SKOV3, and OVCAR4 cells were cultured in serum-free media in the Ultra-Low Attachment dishes for 12 days to enrich CSCs. The cellular ROS level was determined using the DCFDA fluorescent probe by FACS. (**A**) Representative FACS histograms of ROS production. (**B**) Relative ROS level was calculated and plotted. N = 3, Bar: SD, **: P < 0.01.

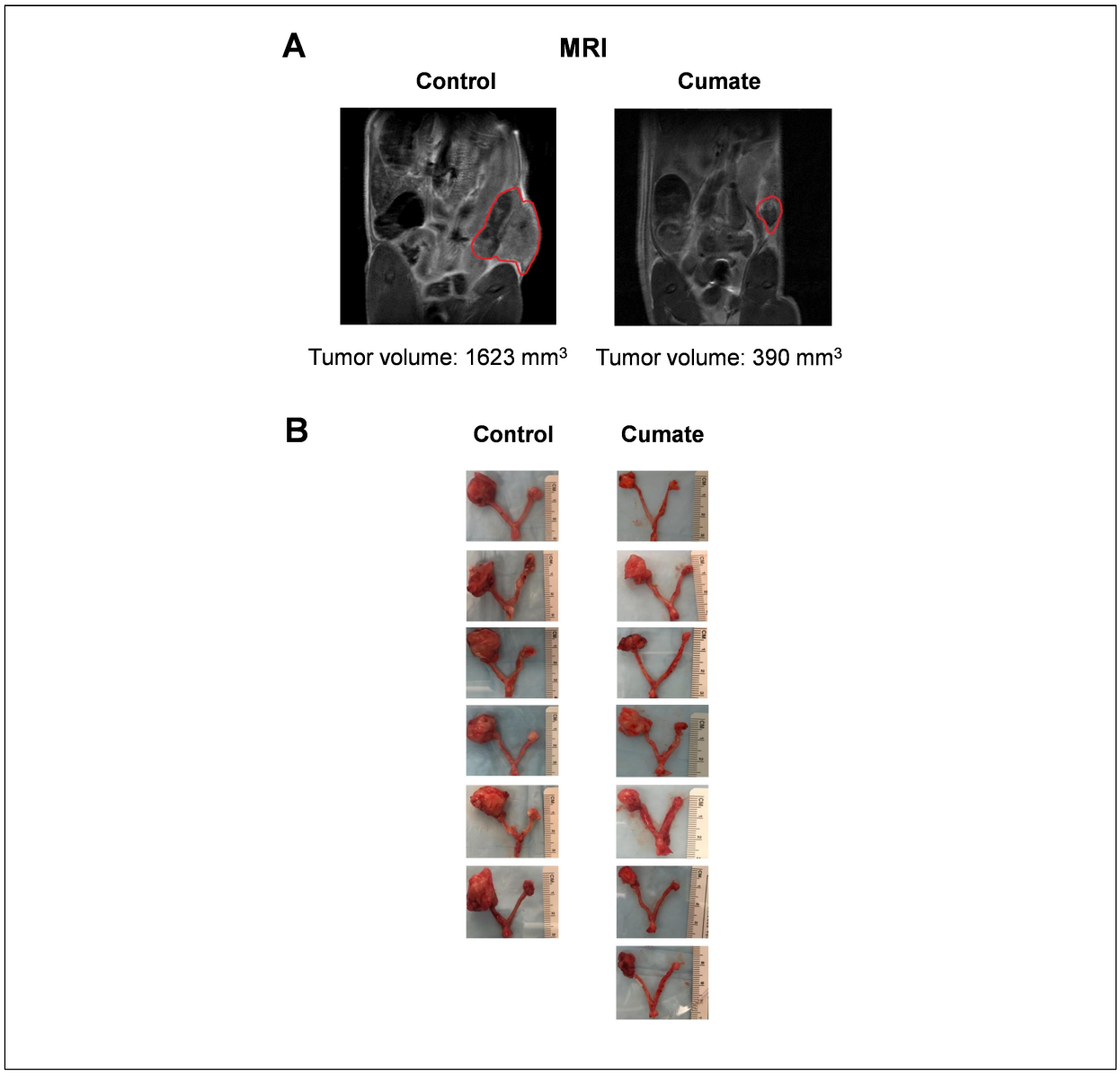
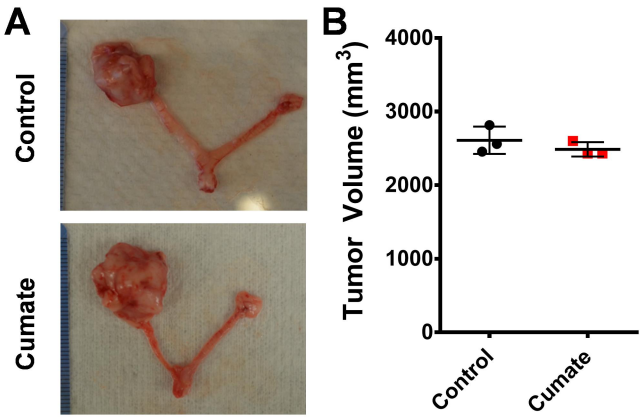


Figure S13 Cumate-induced inhibition of miR-328-3p impeded orthotopic ovarian xenograft growth. 2008-MIZP-328-3p cells were injected into ovary to generate orthotopic xenografts. Mice were injected with PBS or cumate once every other day for 14 days. MRI was conducted to determine the tumor volumes (A). Xenografts were removed after 14 days (B).



Group	No. of mice with metastasis
Control	3(3)
Cumate	2(3)
P value	0.74

Note: Total No. of mice are In parenthesis

Figure S14. Cumate does not affect tumor growth and metastasis in orthotopic ovarian xenograft model. OV2008 cells ($1X10^5$) were injected into ovary to generate orthotopic xenografts. Mice were injected with PBS (3 mice) or cumate (3 mice) once every other day for 14 days. (**A)** Representative image of orthotopic ovarian xenografts in mice without or with cumate treatment. (**B)** The tumor volume of xenografts in two groups. (**C)** Macroscopic appearances and counts of ovarian metastasis nodules in peritoneal cavity.

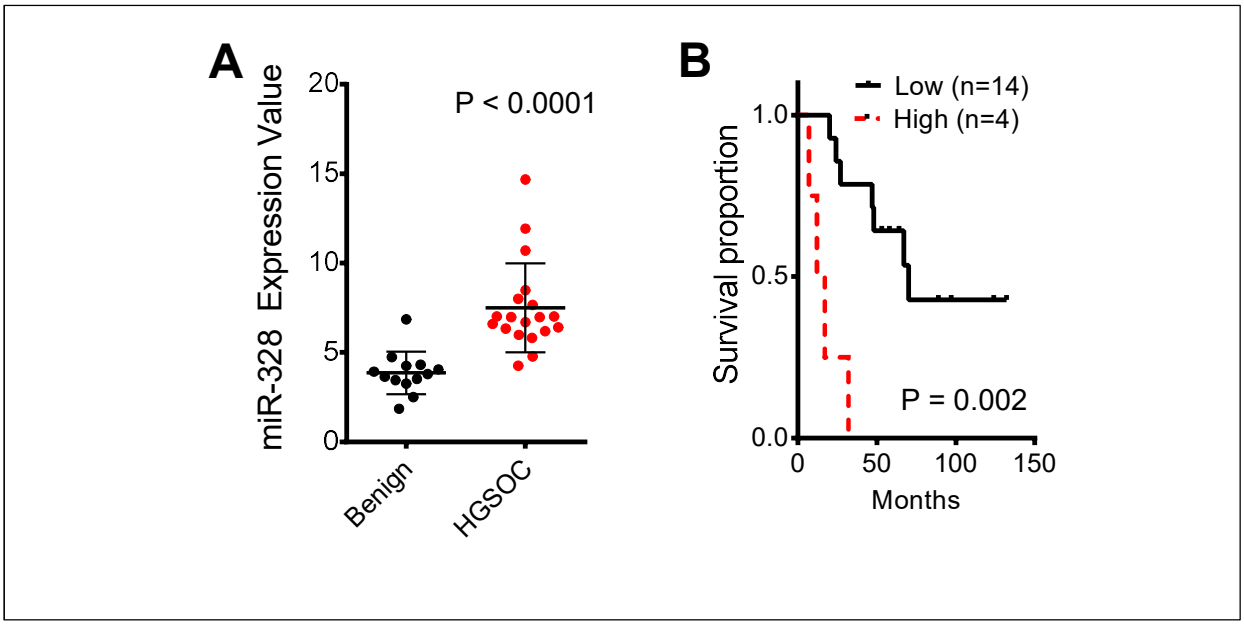


Figure S15. Expression of miR-328-3p and its prognostic significance in ovarian cancer. The miRNA expression data in 19 epithelial ovarian cancer tissues and 13 benign ovarian masses from GSE81873 were re-analyzed to show the miR-328 expression level in these samples (A). Eighteen patients were divided into two groups with either high (>80th percentile) or low expression (<80th percentile) of miR-328. Kaplan-Meier survival analysis was conducted to estimate the relationship between miR-328-3p expression and survival (B).