Gynecologic Oncology 153 (2019) 651–660

Contents lists available at ScienceDirect

Gynecologic Oncology

journal homepage: www.elsevier.com/locate/ygyno

SOX2 and SOX9 are markers of clinically aggressive disease in metastatic high-grade serous carcinoma

Miriam Sherman-Samis [a], Hadil Onallah [a], Arild Holth [b], Reuven Reich [a,*,1], Ben Davidson [b,c,**]

[a] Institute of Drug Research, School of Pharmacy, Faculty of Medicine, The Hebrew University of Jerusalem, Jerusalem 91120, Israel
[b] Department of Pathology, Oslo University Hospital, Norwegian Radium Hospital, N-0310 Oslo, Norway
[c] University of Oslo, Faculty of Medicine, Institute of Clinical Medicine, N-0316 Oslo, Norway

HIGHLIGHTS

- Higher SOX2 and SOX9 expression in HGSC effusions is associated with primary chemoresistance and shorter survival.
- Nanog is secreted from HGSC cells into exosomes in effusion supernatants.
- Nanog knockout in vitro suppresses reduced migration, invasion and MMP9 activity.

ARTICLE INFO

Article history:
Received 6 November 2018
Received in revised form 27 February 2019
Accepted 10 March 2019
Available online 21 March 2019

Keywords:
Cancer stem cells
High-grade serous carcinoma
Effusion
Exosomes
Chemotherapy response
Survival

ABSTRACT

Objective. The aim of this study was to analyze the expression, biological role and clinical relevance of cancer stem cell markers in high-grade serous carcinoma (HGSC).

Methods. mRNA expression by qRT-PCR of *NANOG*, *OCT4*, *SOX2*, *SOX4*, *SOX9*, *LIN28A* and *LIN28B* was analyzed in 134 HGSC specimens (84 effusions, 50 surgical specimens). Nanog, OCT3/4, SOX2 and SOX9 protein expression by immunohistochemistry was analyzed in 52 HGSC effusions. Nanog protein expression in exosomes from 80 HGSC effusions was studied by Western Blotting. OVCAR3 cells underwent CRISPR/Cas9 Nanog knockout (KO), and the effect of Nanog KO on migration, invasion, proliferation and proteolytic activity was analyzed in OVCAR3 and OVCAR8 cells.

Results. OCT4 mRNA was overexpressed in effusions compared to solid specimens ($p = 0.046$), whereas *SOX9* was overexpressed in the ovarian tumors compared to effusions and solid metastases ($p = 0.003$). Higher *SOX2* and *SOX9* expression was associated with primary (intrinsic) chemoresistance ($p = 0.009$ and $p = 0.02$, respectively). Higher *SOX9* levels were associated with shorter overall survival in univariate ($p = 0.04$) and multivariate ($p = 0.049$) analysis. OCT3/4, SOX2 and SOX9 proteins were found in HGSC cells, whereas Nanog was detected only in exosomes. Higher SOX2 protein expression was associated with shorter overall survival in univariate analysis ($p = 0.049$). OVCAR cells exposed to OVCAR3 *NANOG* KO exosomes had reduced migration, invasion and MMP9 activity.

Conclusions. SOX2 and *SOX9* mRNA levels in HGSC effusions may be markers of clinically aggressive disease. Nanog is secreted in HGSC exosomes in effusions and modulates tumor-promoting cellular processes in vitro.

1. Introduction

Ovarian cancer, consisting mainly of ovarian carcinoma (OC), is the gynecologic malignancy with the highest case to fatality ratio, mainly due to diagnosis at advanced stage (FIGO stage III/IV) [1]. Chemoresistance, either intrinsic or acquired in the course of disease progression, is an additional factor contributing to this poor outcome [2]. OC, and particularly its most common histologic type, high-grade serous carcinoma (HGSC), has strong predilection to metastasis within the abdominal cavity, characteristically forming both solid lesions and malignant ascites [3]. OC cells in ascites constitute a chemoresistant cell population with an important role in promoting tumor progression and fatal outcome in this cancer [2,4].

Cancer stem cells (CSC) represent a small population of cells during the initial tumor growth. Chemotherapy will often eradicate the majority of tumor cells, but is ineffective in eliminating CSC which, with time, proliferate and are the origin of disease recurrence. Several postulated

* Correspondence to: R. Reich, Institute for Drug Research, School of Pharmacy, Faculty of Medicine, The Hebrew University of Jerusalem, Jerusalem 91120, Israel.
** Correspondence to: B. Davidson, Department of Pathology, Norwegian Radium Hospital, Oslo University Hospital, Montebello, N-0310 Oslo, Norway.
E-mail addresses: reuvenr@ekmd.huji.ac.il (R. Reich), bend@medisin.uio.no (B. Davidson).
[1] R.R. is affiliated with the David R. Bloom Center for Pharmacy and the Adolf and Klara Brettler Center for Research in Molecular Pharmacology and Therapeutics at The Hebrew University of Jerusalem, Israel.

https://doi.org/10.1016/j.ygyno.2019.03.099
0090-8258/

Table 1
Clinicopathologic parameters for HGSC patients with effusion (n = 84), ovarian tumor (n = 30) and solid metastasis (n = 19)[a].

Parameter	Effusions (n = 84)	Ovary (n = 30)	Solid metastasis (n = 19)
Age (mean)	38–83 years (61)	31–82 years (59)	50–86 years (67)
FIGO stage			
I	0	3	0
II	2	1	0
III	46	22	13
IV	36	4	4
NA	0	0	2
Residual disease[b]			
0 cm	14	14	1
≤1 cm	29	10	6
>1 cm	32	3	9
NA	9	3	3
CA 125 at diagnosis (range; median)[c]	11–43,800 (800)	178–28,000 (1114)	8–3741 (892)
Chemoresponse after primary treatment			
CR	36	23	8
PR	25	3	3
SD	7	0	3
PD	10	1	3
NA	6	3	2

Abbreviations: NA = not available; CR = complete response; PR = partial response; SD = stable disease; PD = progressive disease.

[a] Three patients with both ovarian tumor and solid metastasis are represented in both columns. One patient with 2 solid metastases is listed only once in the metastasis column.

[b] Values for 53 patients who received surgery as upfront treatment were as follows: 0 cm: 6 patients; ≤1 cm: 23 patients; >1 cm: 23 patients; unknown: 1 patient.

[c] Available for 61/84 patients with effusions, 29/30 patients with ovarian tumor and all 19 patients with solid metastasis.

CSC markers have been identified in OC, including surface markers, such as CD24, CD44, CD117 and CD133, and the intracellular cytoplasmic and/or nuclear proteins aldehyde dehydrogenase isoform 1A1 (ALDH1A1), OCT4, Nanog, SOX2, Notch-1, nestin and others. The presence of a side population identified by flow cytometry has been applied as an additional criterion [5,6]. Several of these markers have been identified in OC CSC in ascites in experimental models and/or patient material [7–20]. However, the clinical relevance of these markers has not been assessed in large series of patients with OC effusions. Two recent studies by a member of our group have failed to identify such role for CD24 and nestin in this anatomic compartment [21,22].

The present study assessed the clinical and biological role of the CSC markers in HGSC.

2. Materials and methods

2.1. Cell lines and reagents

The OVCAR3 and OVCAR8 OC cell lines were obtained from the American Type Culture Collection (ATCC) and cultured according to the manufacturer's instructions. OVCAR3 cells were cultured in DMEM, OVCAR8 in RPMI (Biological Industries, Beit-Haemek, Israel). The medium was supplemented with 1% L-glutamine, 1% sodium pyruvate, 1% vitamin solution, 1% non-essential amino acids (Biological Industries) and 10% fetal calf serum (Sigma-Aldrich, St. Louis MO). Cells were grown in a humidified atmosphere of 95% air and 5% CO2. In cells from which exosomes were extracted, the medium used was EXCELL® Advanced™ CHO Fed-batch Medium (Sigma-Aldrich).

2.2. Patients and specimens

Specimens were submitted for routine diagnostic purposes to the Department of Pathology at the Norwegian Radium Hospital during the period of 1998–2008. HGSC specimens and clinical data were obtained from the Department of Gynecologic Oncology, Norwegian Radium Hospital. As the fallopian tubes have not been adequately assessed in this cohort, tumors in the ovary are specified as such without reference to primary site.

Tumors were diagnosed by an experienced gyn-pathologist and cytopathologist (BD). The diagnosis of HGSC was made based on the combination of morphology (obvious nuclear atypia and the presence of multiple mitoses) and the presence of aberrant (diffusely positive or entirely negative) p53 immunostaining. Frozen sections from all solid tumors were reviewed by the same author, and only specimens with tumor cell population > 50% and minimal or no necrosis were included in this study.

HGSC effusions analyzed using quantitative real-time reverse-transcription polymerase chain reaction (qRT-PCR) consisted of 84 effusions (67 peritoneal, 17 pleural) from 84 patients. Forty-one effusions were tapped at diagnosis and were chemo-naive and 42 were tapped after exposure to chemotherapy. Chemotherapy status was unknown for 1 specimen. The 42 post-chemotherapy specimens included 35 effusions tapped at disease recurrence, 4 effusions sampled in the primary disease setting after administration of neoadjuvant chemotherapy and 3 effusions from patients who only received chemotherapy.

Additionally, 50 solid lesions, including 30 ovarian resections and 20 solid metastases, the majority omental, were analyzed for comparative purposes. The majority of specimens were not patient-matched. However, patient-matched ovarian tumor and solid metastasis were available from 3 patients and 2 metastases from the same patient in an additional case. Clinicopathologic data are presented in Table 1.

Effusions were centrifuged immediately after tapping, and cell pellets were frozen at −70 °C in equal amounts of RPMI 1640 medium (GIBCO-Invitrogen, Carlsbad, CA) containing 50% fetal calf serum (PAA Laboratories GmbH, Pasching, Austria) and 20% dimethylsulfoxide (Merck KGaA, Darmstadt, Germany). Surgical specimens were frozen at −70 °C without any treatment. Additionally, 80 effusion supernatants (59 peritoneal, 21 pleural) collected in the years 1998–2003 from which exosomes were isolated were frozen at −70 °C without any treatment.

Informed consent was obtained according to national and institutional guidelines. Study approval was given by the Regional Committee for Medical Research Ethics in Norway.

2.3. qRT-PCR

cDNA was transcribed of 500 ng total RNA. qRT-PCR was carried out using the KAPA SYBR FAST qPCR kit (Kapa Biosystems, Wilmington MA) according to the manufacturer's protocol. Specificity was confirmed by appropriate melting curves. mRNA levels were established by

Table 2
Primer sequences.

Gene		Primer sequence
NANOG	Forward	5′-GGAGCCTAATCAGCGAGGTT-3′
	Reverse	5′-AGACGGCAGCCAAGGTTATT-3′
OCT4B1	Forward	5′-TCCCTGAACCTAGTGGGGAG-3′
	Reverse	5′-GGTTTCTGCTTTGCATATCTCCT-3′
SOX2	Forward	5′-CAGCGCATGGACAGTTACG-3′
	Reverse	5′-TTCATGTAGGTCTGCGAGCTG-3′
SOX4	Forward	5′-CCTGAACCCCAGCTCAAACT-3′
	Reverse	5′-GATCATCTCGCTCACCTCGG-3′
SOX9	Forward	5′-AGGAAGTCGGTGAAGAACGGG-3′
	Reverse	5′-CCTCTCGCTTCAGGTCAGCC-3′
LIN28A	Forward	5′-ATCAAAAGGAGACAGGTGCTAC-3′
	Reverse	5′-GCAAAAGAATAGCCCCCACC-3′
LIN28B	Forward	5′-TTGATGCAGAAGATCACTCCGT-3′
	Reverse	5′-GGGCTTCCCTCTCGGTTTATC-3′
RPLP0	Forward	5′-CCAACTACTTCCTTAAGATCATCCAACTA-3′
	Reverse	5′-ACATGCGGATCTGCTGCA-3′

calculating the target molecule: reference gene (*RPLP0*) ratio. Primer sequences are listed in Table 2.

2.4. Immunohistochemistry (IHC)

Formalin-fixed, paraffin-embedded sections from the 52 of the 84 HGSC effusions analyzed using qRT-PCR were immunohistochemically analyzed for SOX2, SOX9, OCT3/4, and Nanog expression using the Dako EnVision™ FLEX (OCT3/4 and Nanog) or FLEX+ (SOX2 and SOX9) System (Agilent/Dako, Glostrup, Denmark). Following deparaffinization, sections were incubated with a 0.3% hydrogen peroxide (H2O2) solution for 5 min to block endogenous tissue peroxidase activity. Sections were then incubated with the relevant antibody. Antibody details and staining were as follows:

SOX2 mouse monoclonal antibody (clone 245610; cat#MAB2018; R&D systems, Minneapolis MN): 1:200 dilution, antigen retrieval in citrate buffer (pH 6).

SOX9 mouse monoclonal antibody (clone 3C10; cat#ab76997; Abcam, Cambridge UK): 1:5000 dilution, antigen retrieval in citrate buffer (pH 6).

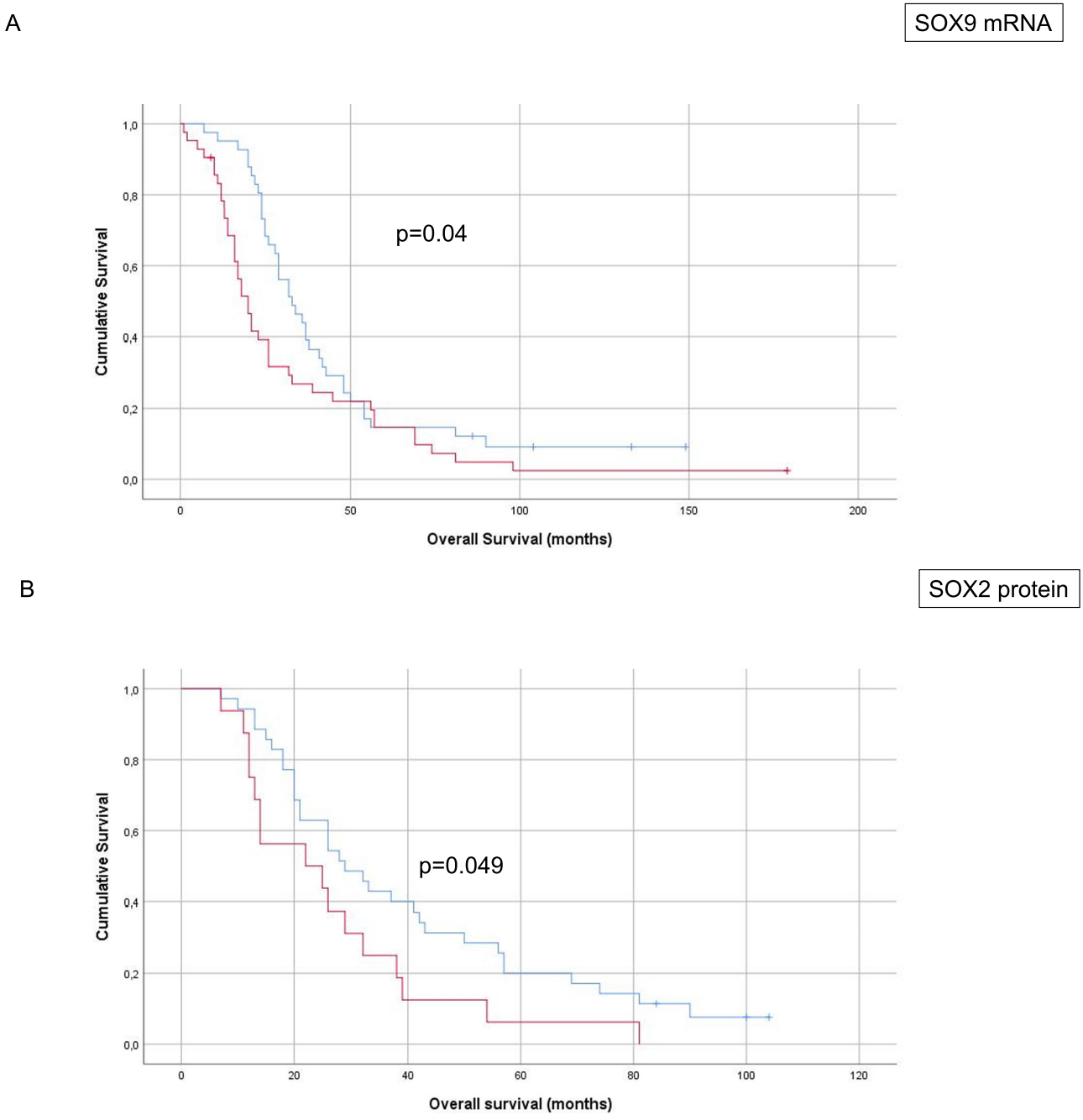


Fig. 1. SOX2 and SOX9 expression in HGSC effusions is associated with shorter survival. A. Kaplan-Meier survival curve showing the association between *SOX9* mRNA expression and overall survival (OS) in HGSC effusions (n = 83; one failed analysis). Patients with effusions with high (above median) *SOX9* mRNA expression levels (n = 42; red line) had mean OS of 32 months compared to 46 months for patients with effusions having low *SOX9* mRNA levels (n = 41, blue line; p = 0.04). B. Kaplan-Meier survival curve showing the association between SOX2 protein nuclear expression and OS in HGSC effusions (n = 51; one missing analysis). Patients with effusions with nuclear SOX2 expression (n = 16; red line) had mean OS of 27 months compared to 40 months for patients with SOX2-negative effusions (n = 35, blue line; p = 0.049).

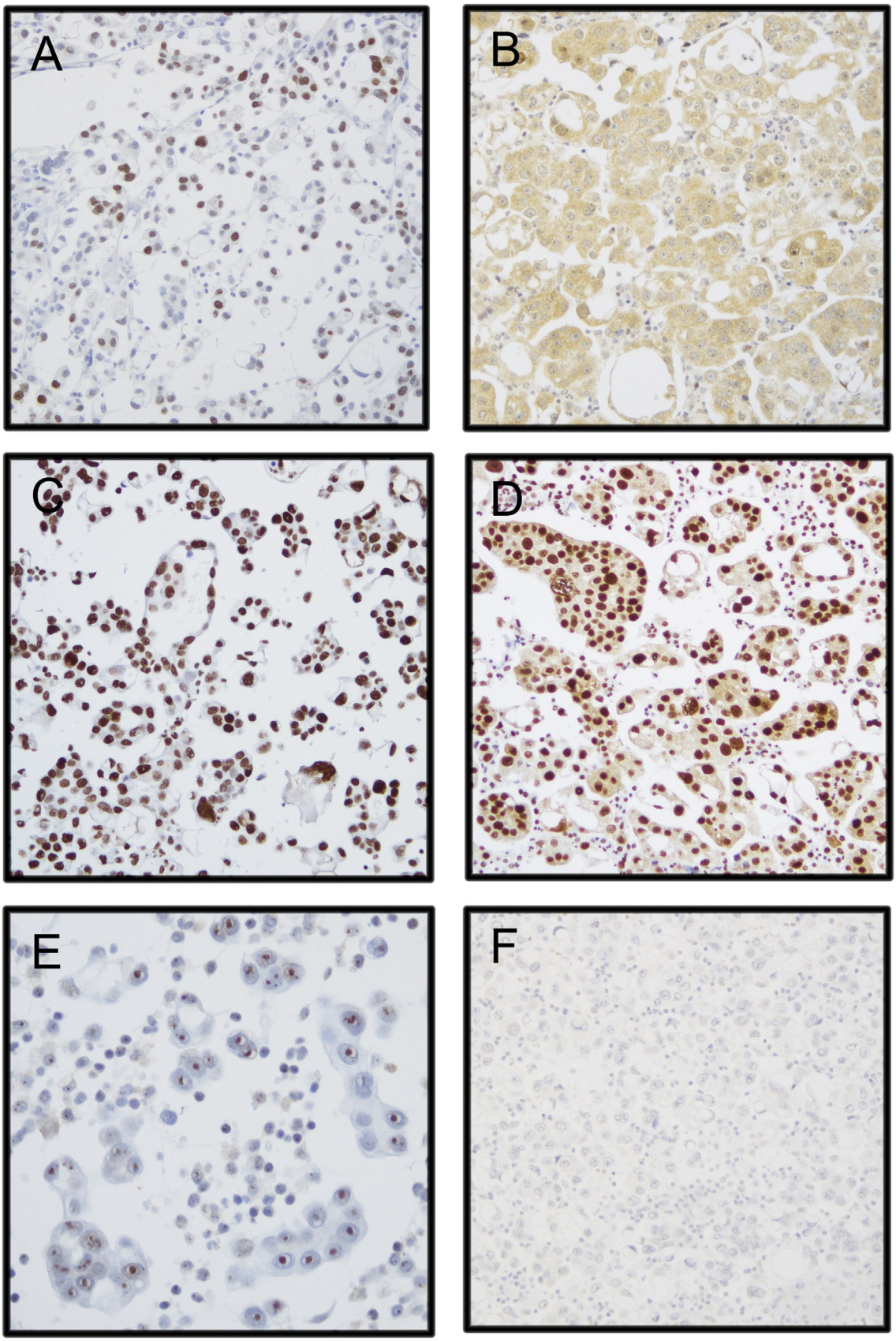


Fig. 2. SOX2, SOX9 and OCT3/4 protein expression in HGSC effusions. (A–B): Two effusions with SOX2 expression in HGSC cells. Expression is nuclear in (A), predominantly cytoplasmic (few positive nuclei) in (B). (C–D): Two effusions with SOX9 expression in HGSC cells. Expression is nuclear in (C), combined nuclear and cytoplasmic in (D). (E): Nuclear OCT3/4 nuclear expression. Staining is seen in nucleoli and chromatin clusters. (F): Negative Nanog staining.

OCT3/4 goat polyclonal antibody (cat# AF1759; R&D systems): 1:400 dilution, antigen retrieval in citrate buffer (pH 6).

Nanog goat polyclonal antibody (cat# AF1997; R&D systems): 1:200 dilution, antigen retrieval in citrate buffer (pH 6).

Sections were thereafter treated with EnVision™ Flex+ mouse or goat linker (15 min) and EnVision™ Flex/HRP enzyme (30 min), stained for 10 min with 3,3 diaminobenzidine tetrahydrochloride (DAB), counterstained with hematoxylin, dehydrated and mounted in Richard-Allan Scientific Cyto seal XYL (Thermo Fisher Scientific). Positive controls consisted of normal testis. Negative controls were stained with nonrelevant antibody of the same isotype for monoclonal antibodies and normal goat serum for polyclonal antibodies.

2.5. Western blotting (WB)

Cells and exosomes were lysed with 1% NP-40, 20 mM Tris-HCl (pH 7.5), 137 mM NaCl, 0.5 mM EDTA (Mallinckrodt Baker Inc., St. Louis MO), 10% glycerol (Frutarom LTD, Haifa, Israel), 1% protease inhibitor cocktail (Sigma-Aldrich) and 0.1% SDS (Biological Industries). After centrifugation, protein content was quantified using the Bradford assay, and 25 μg of protein from each specimen were loaded onto 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels. The separated extracts were transferred onto PVDF membrane (Millipore, Bedford, MA). In order to block nonspecific binding, membranes were incubated for 1 h in 5% low fat milk dissolved in TBST. Membranes containing proteins originating from exosomes did not undergo blocking. Membranes were then incubated with the following antibodies: Goat anti-Nanog polyclonal antibody (catalog # AF1997, R&D Systems, Minneapolis MA), rabbit anti-CD63 polyclonal antibody (catalog # SC15363, Santa Cruz Biotechnology, Santa Cruz CA), and rabbit anti-GAPDH monoclonal antibody (catalog # 14C10, Cell Signaling Biotechnology, Beverly MA).

GAPDH was used as loading control for proteins originating from cells and CD63 was used as a loading control for proteins originating from exosomes. Proteins were detected using EZ-ECL Chemiluminescence detection kit for HRP (Biological Industries). Densitometer analysis of films was performed using a computerized image analysis program (Image-J, NIH, Bethesda MD).

2.6. CRISPR/Cas9 knockout (KO)

The vector was digested by the BBSI restriction enzyme (New England Biolabs, Ipswich MA). Extraction and purification of digested plasmid from agarose gel of PCR products was done with Nucleospin® Gel and PCR Clean-up Kit (Macherey-Nagel, Düren, Germany). Ligation was performed with T4 DNA ligase (New England Biolabs). Specific single guide RNA (sgRNA) inserts were designed with the help of Zhang Lab scoring (http://crispr.mit.edu/) and were targeted at the beginning of the 5′ of the *NANOG* gene:

Forward: CACCTGTCGCAAAAAAGGAAGACA

Reverse: AAACTGTCTTCCTTTTTTGCGACA

The insert selected targeted Exon 2 of Nanog in order to stop early translation. The inserts were 5′ phosphorylated and annealed on a ramp between 95 and 25 °C. The plasmid (pSpCas9 BB -2A- GFP, PX458 plasmid # 48138 vector) was a generous gift from the lab of Prof. Yehudit Bergman at the Hebrew University of Jerusalem. The plasmid was digested by the BBSI restriction enzyme. Ligation was performed with T4 ligase. The plasmids were then transformed to competent DHα5 *E. coli* bacteria. Plasmids were extracted with a commercial kit, GeneJet Plasmid Miniprep (Fermentas Life Sciences/Thermo Fisher, Waltham MA) and sent to sequencing in order to confirm the insertion of the sgRNA. Thereafter, the plasmids were transfected into OVCAR3 cells with Lipofectamine 3000 (Invitrogen, Carlsbad CA). Sorting by FACS Aria II (Ein Karem, Hebrew University) was performed and the GFP positive sorted cells were seeded as single cell colonies, which resulted as single cell clone KO cells, subsequently analyzed for KO by WB analysis.

2.7. siRNA

SOX2 and SOX9 were silenced using the following siRNAs:

SOX2: mix of SASI_Hs01_00050572, SASI_Hs01_00050573, SASI_Hs01_00050580

SOX9: EHU021061

siRNAs were from Sigma-Aldrich and were used according to the manufacturer's instructions.

2.8. Exosome extraction and quantitation

2.8.1. Clinical specimens

Exosomes were extracted from 250 μl of effusion fluid according to the user's manual of ExoQuick-TC (System Biosciences, Mountain View CA) and quantified with the Bradford assay. Approximately 27 and 100 whole exosomes were tested for protein and mRNA, respectively.

2.8.2. Cell line

1×10^7 cells were seeded and cultured in serum-free medium, BSA 0.1% for 24 h. Conditioned medium was filtered with a 0.1 μM PVDF (Merck Millipore, Tullagreen, Ireland) filter and then concentrated with 3000 MWCO vivaspin 20 (Santorius, Göttingen, Germany). Exosomes were extracted from the supernatant according to the ExoQuick-TC manual. The resulting pellet containing exosomes was re-suspended in 100 μl PBS and analyzed for protein concentration by the Bradford assay.

2.9. Scratch assay

OVCAR3, Nanog KO-C and Nanog KO-E cells (400,000) were seeded in 6-well plates. They were treated with 10 μg OVCAR3 exosomes and incubated for 24 h in DMEM serum-free medium, BSA 0.1%. Prior to assay the cells were washed with PBS and replaced with DMEM BSA 0.1% without exosomes. Each well was scratched twice with a sterile tip and imaged at $t = 0$, $t = 6$ and $t = 24$ h. The closure of the wound was analyzed by T-scratch software51.

2.10. Proliferation assay

OVCAR8, OVCAR3, Nanog KO-C and Nanog KO-E cells (400,000) were seeded in 6-well plates and were treated with 10 μg OVCAR3, KO-C and KO-E exosomes for 24 h in serum-free medium, BSA 0.1%. At 24 h, cells were treated with 0.5 mg/ml 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; EMD) for 30 min. Cells were then lysed with DMSO (Merck) and the absorbance of the solution was read at 560 nm using Multiscan RC (Thermo Fisher).

Table 3
IHC results in 52 HGSC effusions.

Antibody	Localization	Staining extent				
		0%	1–5%	6–25%	26–75%	75–100%
SOX2[a]	Nucleus	35	11	2	2	1
	Cytoplasm	38	0	4	4	5
SOX9	Nucleus	0	0	0	3	49
	Cytoplasm	36	4	0	3	9
OCT3/4	Nucleus	50	2	0	0	0
	Cytoplasm	22	2	6	12	10
Nanog	Any	52	0	0	0	0

[a] One missing case.

2.11. Invasion assay-Boyden chamber

OVCAR8, OVCAR3, Nanog KO-C and Nanog KO-E cells (400,000) were seeded in 6-well plates and were treated with 10 μg OVCAR3, KO-C and KO-E exosomes for 24 h in serum-free medium, BSA 0.1%. Cells were than seeded on 8.0 μM PVDF filters (GE Whatman, Little Chalfont, Buckinghamshire, UK) coated with 25 μg Matrigel in Boyden chambers. On the opposite side, a chemoattractant (conditioned medium from the 3 T3 fibroblast cell line) was placed. After 6 h of incubation in optimal conditions, filters were removed and the presence of invading cells was determined by staining and counted.

2.12. Zymography

OVCAR8 cells (400,000) were seeded in 6-well plates. They were treated with 10 μg OVCAR3, KO-C and KO-E exosomes and incubated for 24 h in serum-free medium, BSA 0.1%. Samples from the supernatants were collected after 24 h and were analyzed for collagenolytic activity, determined on 1 mg gelatin/ml, 10% SDS-PAGE gel. Bands were analyzed by ImageJ software.

2.13. Statistical analysis

Statistical analysis was performed applying the SPSS-PC package (Version 25, Chicago IL). Probability of <0.05 was considered statistically significant. Comparative analysis of CSC marker expression in effusions, ovarian tumors and solid metastases was performed using the Kruskal-Wallis H test. Analysis of the association between expression levels of these molecules in HGSC effusions and clinicopathologic parameters was executed using the Mann-Whitney U (for 2 groups) or Kruskal-Wallis H (for 3 groups) test. For this analysis, as well as for survival analysis, clinicopathologic parameters

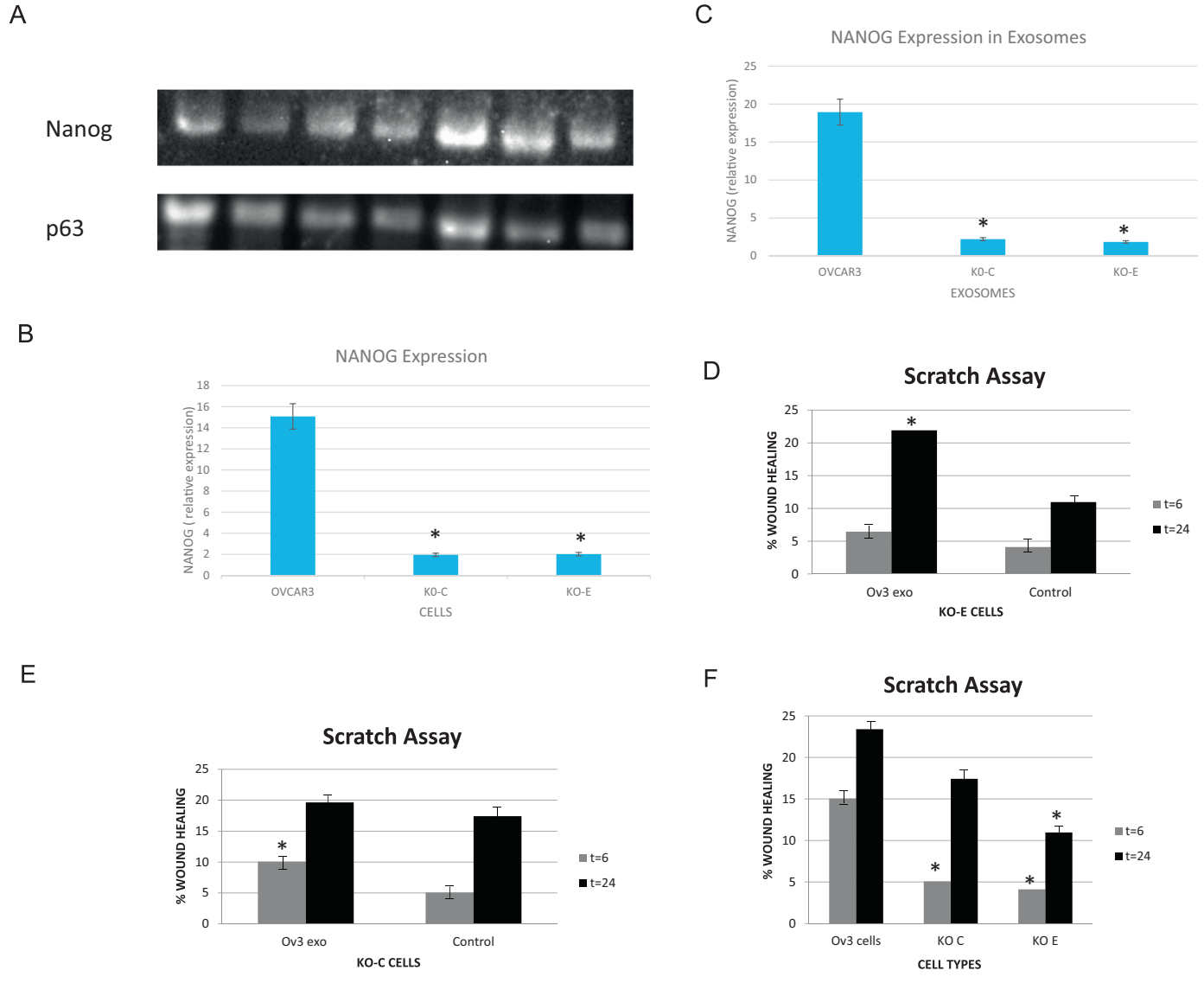
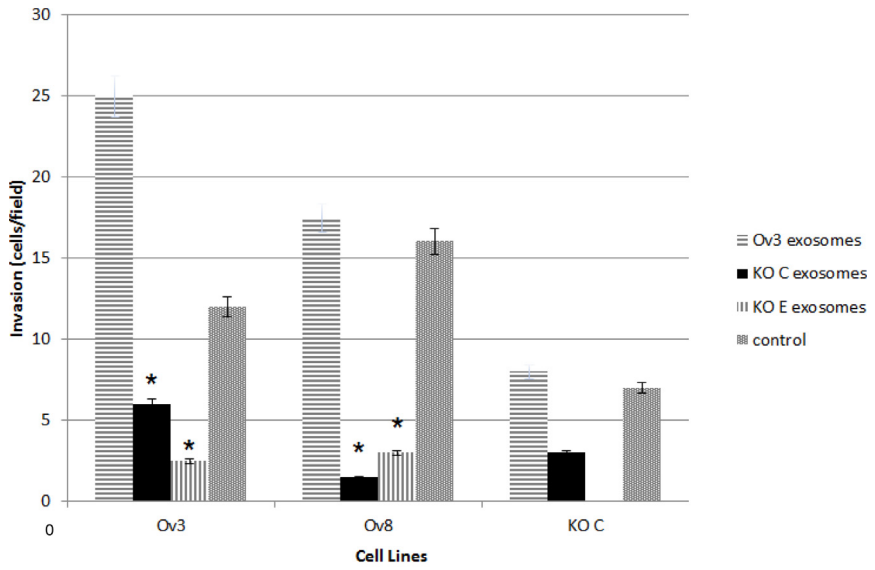
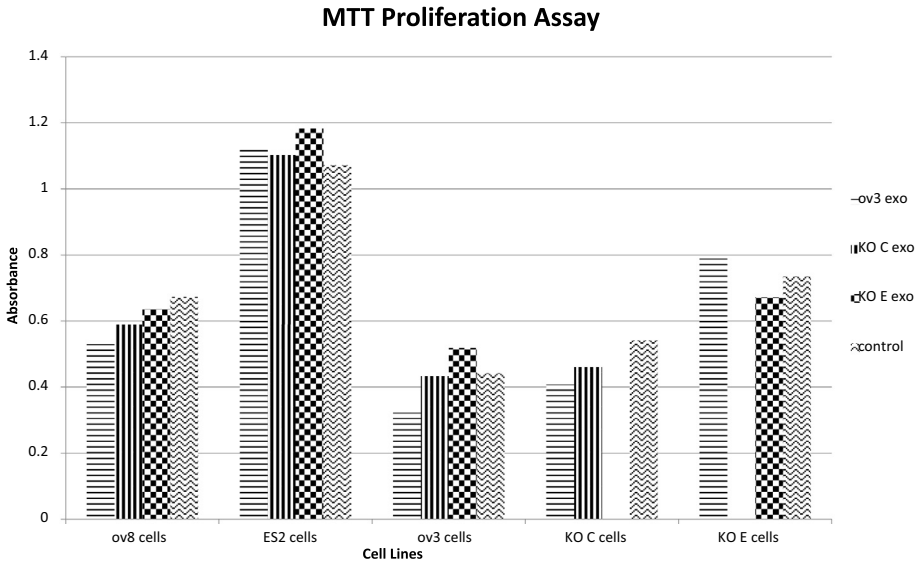


Fig. 3. Nanog in HGSC exosomes and cell lines. -A: Nanog protein expression in effusion-derived exosomes. -B: Nanog protein expression in OVCAR3 cell line and its downregulation in the two KO-Cell lines created; $p < 0.01$ for both cell lines. -C: Nanog protein expression in OVCAR3 exosomes and its downregulation in the two KO-exosomes created; $p < 0.01$ for both cell lines. -D–F: Scratch assay. -D: Graph depicting % of wound closure in KO-E cell lines treated with OVCAR3 exosomes and untreated cells at 6 and 24 h; $p < 0.01$. -E: Graph depicting % wound closure in KO-C cell lines treated with OVCAR3 exosomes and untreated cells at 6 and 24 h; $p < 0.05$. -F: Graph depicting % of wound closure in OVCAR3, KO-C and KO-E cells without any treatment at 6 and 24 h. -G: Invasion Assay. Treatment with Nanog KO-exosomes reduces invasion ($p < 0.05$). -H: MTT proliferation assay of OVCAR8, ES2, OVCAR3, KO-C and KO-E cell lines treated with OVCAR3, KO-C and KO-Exosomes. Proliferation is not significantly affected ($p > 0.05$). -I: MMP9 activity. Nanog KO induces a slight decrease MMP9 activity, evidenced as reduced ability of tumor cells to degrade gelatin. -J: Invasion Assay. SOX2 and SOX9 siRNA significantly reduces invasion ($p < 0.05$). -K: Wound healing assay. SOX2 and SOX9 siRNA significantly reduces motility ($p < 0.05$). -L: MMP2 activity. SOX2 and SOX9 siRNA significantly reduces gelatinolytic activity ($p < 0.05$).

G



H



I

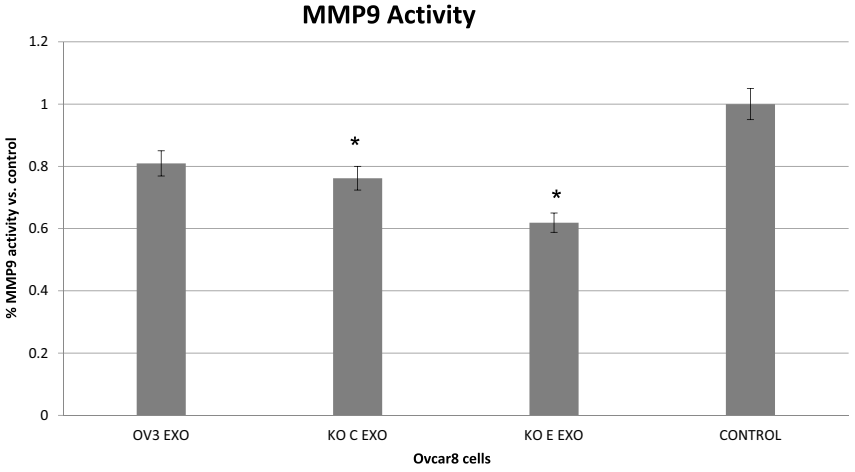
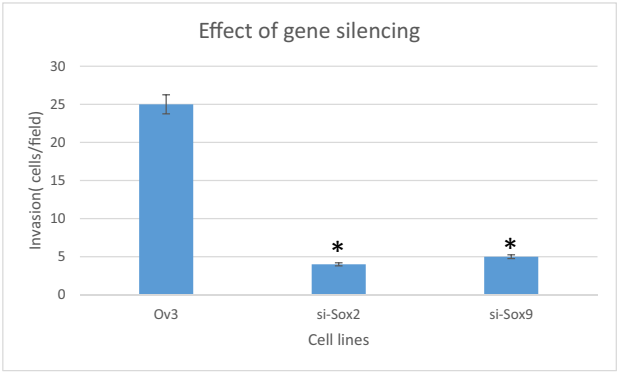
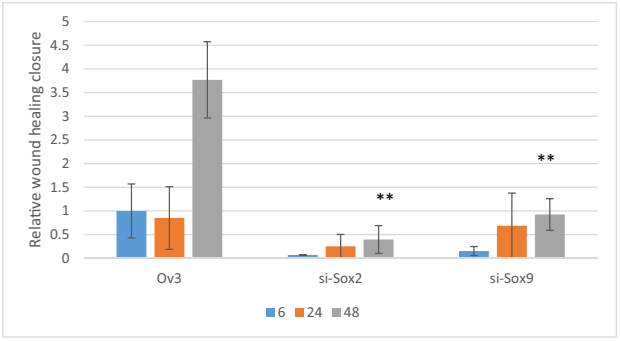


Fig. 3 (*continued*).

J



K



L

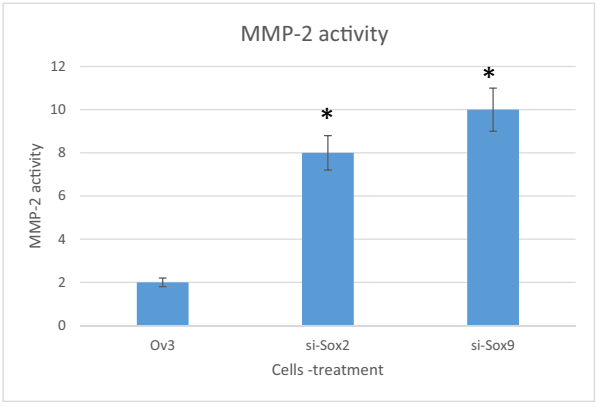


Fig. 3 (*continued*).

were grouped as follows: age: ≤60 vs. >60 years; effusion site: peritoneal vs. pleural; FIGO stage: III vs. IV; chemotherapy status: pre- vs. post-chemotherapy specimens; residual disease (RD): 0 cm vs. ≤1 cm vs. >1 cm; response to chemotherapy: complete response vs. partial response/stable disease/progressive disease.

Progression-free survival (PFS) and overall survival (OS) were calculated from the date of the last chemotherapy treatment/diagnosis to the date of recurrence/death or last follow-up, respectively. Univariate survival analyses of PFS and OS were executed using the Kaplan-Meier method and log-rank test. Platinum resistance was defined as PFS ≤ 6 months according to guidelines published by the Gynecologic Oncology Group and progressive disease or recurrence was evaluated by RECIST criteria. Multivariate survival analysis was performed using the Cox regression model (Enter function).

Analysis KO metastatic assays were performed using a two-tailed student *t*-test.

3. Results

3.1. SOX9 and OCT4 are differentially expressed at different anatomic sites in HGSC

Comparative analysis of *SOX2*, *SOX9*, *NANOG*, *OCT4* and *LIN28B* mRNA expression in the ovarian carcinomas, solid metastases and effusions showed significantly higher expression of *OCT4* mRNA in effusions compared to both groups of solid specimens ($p = 0.046$), whereas *SOX9* was overexpressed in the ovarian tumors compared to both effusions and solid metastases ($p = 0.003$). No significant anatomic site-related differences were observed for the 3 remaining CSC markers. *SOX4* and *LIN28A* were not detected in the studied specimens.

3.2. Association with clinicopathologic parameters and survival

The clinical relevance of the studied molecules was analyzed in the effusion cohort, which included the largest number of patients. *OCT4* mRNA levels were significantly higher in pleural effusions compared to peritoneal specimens ($p = 0.03$). Higher *SOX2* and *SOX9* expression was significantly related to intrinsic chemoresistance (PFS ≤ 6 months; $p = 0.009$ and $p = 0.02$, respectively), and showed a trend towards higher expression in patients with poor chemoresponse to first-line chemotherapy ($p = 0.077$ and $p = 0.088$, respectively).

CSC marker expression was unrelated to previous exposure to chemotherapy, patient age, FIGO stage or RD volume ($p > 0.05$; data not shown).

The follow-up period for the 84 patients with HGSC effusions ranged from 1 to 179 months (mean = 37 months, median = 26 months). PFS ranged from 0 to 81 months (mean = 10 months, median = 6 months). At the last follow-up, 78 patients were dead of disease, 3 were alive with disease and 1 was with no evidence of disease. One patient died of treatment complications and 1 was lost to follow-up. The association between CSC marker expression, as well as clinical parameters (age, FIGO stage and RD volume), and survival was analyzed.

In univariate survival analysis of all cases, higher *SOX9* levels were associated with shorter OS ($p = 0.04$; Fig. 1-A). None of the other CSC markers or clinical parameters was significantly associated with survival. Parameters with p-value <0.2, including *NANOG* levels ($p = 0.172$) and patient age ($p = 0.109$), were entered into the Cox multivariate analysis with *SOX9*. Higher *SOX9* levels ($p = 0.049$) and older age ($p = 0.04$) were independent prognostic markers in this analysis.

In separate survival analysis for patients with pre-chemotherapy effusions tapped at diagnosis and patients with post-chemotherapy specimens, a trend for worse OS was observed for *SOX9* levels in pre-chemotherapy effusions ($p = 0.053$), with no other findings (data not shown).

3.3. SOX proteins and OCT3/4 proteins, but not Nanog, are expressed in HGSC effusions

IHC analysis of HGSC effusions showed universal expression of SOX9 protein, particularly at the tumor cell nuclei, with more variable SOX2 and OCT3/4 protein expression, while Nanog was uniformly absent in the 52 studied specimens (Fig. 2; Table 3). Higher cytoplasmic SOX9 expression was significantly related to intrinsic chemoresistance (PFS ≤ 6 months; $p = 0.015$). Higher SOX2 protein expression was associated with shorter OS in univariate analysis ($p = 0.049$). Multivariate analysis was not performed as none of the clinical parameters was associated with OS ($p > 0.05$; data not shown).

In view of the unexpected absence of Nanog from HGSC cells we investigated the possibility that this molecule was localized to an extracellular compartment in HGSC effusions. In agreement with this hypothesis, *NANOG* mRNA was found in exosomes in 71/80 (89%) specimens, and Nanog protein was found in all specimens in analysis of 24 effusion exosome preparations by WB (Fig. 3-A). *SOX2*, *OCT4* and

LIN28 mRNA was not found in HGSC exosomes. *NANOG* mRNA levels in exosomes were unrelated to clinicopathologic parameters or to survival ($p > 0.05$; data not shown).

3.4. CRISPR Cas9 KO in OVCAR3 cells

Two Nanog KO (KO-C and KO-E) lines were created in OVCAR3 cells using the CRISPR/Cas9 method. Exosomes were extracted from OVCAR3 and Nanog KO cell lines. In order to ascertain the Nanog KO protein levels, cells and exosomes were analyzed by Western blot (Fig. 3-B, -C).

In scratch assay, OVCAR3 KO cells had reduced ability to migrate (Fig. 3-D, -E). When KO cells were treated with normal OVCAR3 exosomes, their ability to migrate was partially restored (Fig. 3-F).

Treatment of OVCAR8 and OVCAR3 cells with KO-C and KO-E exosomes significantly reduced the ability of tumor cells to degrade and invade matrigel (Fig. 3-G; $p = 0.005$ and $p = 0.011$, respectively). In each cell line, when treated with OVCAR3 exosomes, the ability to infiltrate the matrigel surpassed the control cells.

The MTT proliferation assay showed no significant change in cell viability following Nanog KO exosomal treatment (Fig. 3-H).

In the zymography assay, MMP9 activity was reduced in OVCAR8 cells treated with KO-C and KO-E exosomes ($p < 0.05$). The enzymatic activity was partially restored by treating OVCAR8 cells with OVCAR3 exosomes (Fig. 3-I).

A 70% silencing of SOX2 and SOX9 was observed after 48 h. SOX2 and SOX9 silencing significantly reduced invasion in Matrigel-coated filter in a Boyden chamber system, motility in wound healing assay, and MMP activity in gelatin impregnated SDS gels (Fig. 3-J to -L). Proliferation was unaffected (data not shown).

4. Discussion

The expression of CSC markers in OC ascites is well-documented. However, changes in their levels as function of anatomic site and their clinical relevance in patients with HGSC effusions are not fully elucidated to date.

The present study analyzed mRNA expression of 7 CSC markers, of which only 5 (*SOX2*, *SOX9*, *NANOG*, *OCT4* and *LIN28B*) were detected in HGSC specimens. *OCT4* mRNA was moderately overexpressed in effusions compared to solid specimens and its presence was confirmed at the protein level. *SOX2* mRNA levels were comparable at all anatomic sites, whereas *SOX9* mRNA levels were highest in the ovarian tumors. However, both proteins were demonstrated in HGSC cells in effusion specimens. Unexpectedly, Nanog protein was absent from HGSC effusions despite the presence of its mRNA, but the protein was detected in HGSC exosomes from the effusion specimens.

Siu et al. analyzed a series of 97 OC and reported on higher Nanog protein expression by IHC in carcinomas of serous type, high grade and low chemosensitivity, Nanog was further identified as an independent prognostic factor of OS and disease-free survival. Stable knockdown of Nanog in OC cell lines suppressed proliferation, migration and invasion, with increased mRNA expression of E-cadherin, caveolin-1, FOXO1, FOXO3a, FOXJ1 and FOXB1, whereas the opposite was observed when Nanog was ectopically overexpressed [23]. Data are nevertheless limited with respect to OC effusions.

Hu and co-workers detected Nanog in OC ascites. However, their study included a single specimen and cells were cultured rather than analyzed in their native state [8]. Wintzell et al. identified this protein in OC ascites, with higher expression in cells growing in spheroids compared to those growing as monolayer [13], and our data are consequently not in agreement with this study. Nanog was detected in rare cells in ascites in the study by Di et al., but the number of specimens analyzed for this marker is unclear [15]. Recently, Yamamoto and coworkers reported that Nanog levels are significantly higher in extracellular vesicles from HGSC ascites compared to benign peritoneal fluid [24], a finding well in agreement with our observation that Nanog is localized to exosomes in these specimens. Data generated from our silencing experiments suggests that Nanog in exosomes regulates invasion, migration and possibly protease activation, in HGSC, while having no effect on proliferation.

Analysis of the clinical relevance of CSC marker expression in HGSC effusions identified SOX2 and SOX9 as candidate markers of poor chemoresponse and shorter survival, and silencing of these 2 genes in OC cells suppressed invasion, migration and proteolytic activity. Data regarding the clinical role of SOX9 in OC is limited to date. However, our findings are in agreement with the observed association between expression of this marker and poor outcome in primary carcinomas of different histotypes, the majority of serous type [25]. Of note, in the latter study, SOX9 was shown to interact with the promoter of *TUBB3*, the gene encoding for class III β-tubulin, a protein whose expression in OC effusions we reported to be associated with chemoresistance and poor survival [22].

Data supporting the clinical role of SOX2 in OC are available from several studies. In the study by Bareiss et al., SOX2 expression increased the expression of other CSC markers and tumor formation of spheres in OC cells, and promoted tumorogenicity in vivo. SOX2 did not affect proliferation, but mediated apoptosis resistance following chemotherapy and TRAIL treatment [26]. *SOX2* amplification was associated with poor survival in analysis of the TCGA dataset [27].

Zhang et al. analyzed 540 carcinomas, the majority HGSC, for SOX2 protein expression using IHC. SOX2 was expressed in 79 tumors (15%), most often in HGSC and carcinosarcoma, and was associated with shorter disease-free survival in univariate, though not multivariate survival analysis [28]. In the study by Wen et al., SOX2 was overexpressed in SKOV3 spheroids compared to monolayers. SOX2 knockdown in SKOV3 and HO8910 spheroids reduced spheroid formation, proliferation, migration, resistance to cisplatin, tumorigenicity in mice, and the expression of CSC and EMT-related genes, whereas SOX2 overexpression had the opposite effects. SOX2 protein expression by IHC was associated with chemoresistance and poor OS and PFS in analysis of 53 tumors specified as type I ($n = 12$) or type II ($n = 41$) [29].

In a single study suggesting association with better outcome, SOX2 protein expression by IHC was associated with longer disease-free survival for patients with stage II-IV OC, with no such role for SOX2 amplification [30].

Of note, SOX2 mutation was found in fallopian tube epithelium with normal morphology in a patient with HGSC, and overexpression of SOX2 protein was commonly observed in fallopian tube epithelium with normal morphology in patients with HGSC and in *BRCA1/BRCA2* mutation carriers who underwent prophylactic salpingo-oophorectomy, while being much less common in fallopian tubes from patients with benign conditions, suggesting that expression of this CSC marker may be an early event in the development of HGSC [31].

In conclusion, our data suggest an association between SOX2 and SOX9 expression at the mRNA and protein level and aggressive clinical behavior in HGSC metastases to serous effusions. Nanog may mediate disease progression via signals generated from exosomes.

Financial acknowledgment

This work was supported by Inger and John Fredriksen Foundation for Ovarian Cancer Research.

Conflict of interest statement

We have no conflict of interest.

Author contributions

MSS: Performed all experiments except immunohistochemistry and SOX silencing and co-wrote the manuscript.

HO: Performed the SOX2 and SOX9 silencing experiments, critically read the revised manuscript.

AH: Performed the immunohistochemistry analysis, critically read the manuscript.

RR: Designed the study, supervised all experiments except immunohistochemistry, critically read the manuscript.

BD: Designed the study, supervised the immunohistochemistry analysis, performed the statistical analysis and co-wrote the manuscript.

References

[1] L.A. Torre, F. Bray, R.L. Siegel, J. Ferlay, J. Lortet-Tieulent, A. Jemal, Global cancer statistics, 2012, CA Cancer J. Clin. 65 (2015) 87–108.

[2] B. Davidson, Recently identified drug resistance biomarkers in ovarian cancer, Expert. Rev. Mol. Diagn. 16 (2016) 569–578.

[3] . Davidson B, Firat P, Michael CW (eds) Serous Effusions. London, UK: Springer, 2nd edition, 2018.

[4] N. Ahmed, K.L. Stenvers, Getting to know ovarian cancer ascites: opportunities for targeted therapy-based translational research, Front. Oncol. 3 (2013) 256.

[5] M. Lupia, U. Cavallaro, Ovarian cancer stem cells: still an elusive entity? Mol. Cancer 16 (2017) 64.

[6] T. Muinao, H.P. Deka Boruah, M. Pal, Diagnostic and prognostic biomarkers in ovarian cancer and the potential roles of cancer stem cells - an updated review, Exp. Cell Res. 362 (2018) 1–10.

[7] Alvero AB, Chen R, Fu HH, et al. Molecular phenotyping of human ovarian cancer stem cells unravels the mechanisms for repair and chemoresistance. Cell Cycle 2009;8:158–66.

[8] L. Hu, C. McArthur, R.B. Jaffe, Ovarian cancer stem-like side-population cells are tumourigenic and chemoresistance, Br. J. Cancer 102 (2010) 1276–1283.

[9] Rizzo S, Hersey JM, Mellor P, et al. Ovarian cancer stem cell-like side populations are enriched following chemotherapy and overexpress EZH2. Mol. Cancer Ther. 2011; 10:325–35.

[10] J. Zhang, X. Guo, D.Y. Chang, D.G. Rosen, I. Mercado-Uribe, J. Liu, CD133 expression associated with poor prognosis in ovarian cancer, Mod. Pathol. 25 (2012) 456–464.

[11] V. Vathipadiekal, D. Saxena, S.C. Mok, P.V. Hauschka, L. Ozbun, M.J. Birrer, Identification of a potential ovarian cancer stem cell gene expression profile from advanced stage papillary serous ovarian cancer, PLoS One 7 (2012), e29079. .

[12] Meng E, Long B, Sullivan P, et al. CD44+/CD24-ovarian cancer cells demonstrate cancer stem cell properties and correlate to survival. Clin. Exp. Metastasis 2012; 29:939–48.

[13] M. Wintzell, E. Hjerpe, E. Åvall Lundqvist, M. Shoshan, Protein markers of cancer-associated fibroblasts and tumor-initiating cells reveal subpopulations in freshly isolated ovarian cancer ascites, BMC Cancer 12 (2012) 359.

[14] Latifi A, Luwor RB, Bilandzic M, et al. Isolation and characterization of tumor cells from the ascites of ovarian cancer patients: molecular phenotype of chemoresistant ovarian tumors. PLoS One 2012;7:e46858.

[15] J. Di, T. Duiveman-de Boer, P.L. Zusterzeel, C.G. Figdor, L.F. Massuger, R. Torensma, The stem cell markers Oct4A, Nanog and c-Myc are expressed in ascites cells and tumor tissue of ovarian cancer patients, Cell. Oncol. (Dordr.) 36 (2013) 363–374.

[16] J. Liao, F. Qian, N. Tchabo, et al., Ovarian cancer spheroid cells with stem cell-like properties contribute to tumor generation, metastasis and chemotherapy resistance through hypoxia-resistant metabolism, PLoS One 9 (2014), e84941. .

[17] K. Abubaker, R.B. Luwor, H. Zhu, et al., Inhibition of the JAK2/STAT3 pathway in ovarian cancer results in the loss of cancer stem cell-like characteristics and a reduced tumor burden, BMC Cancer 14 (2014) 317.

[18] Meng E, Mitra A, Tripathi K, et al. ALDH1A1 maintains ovarian cancer stem cell-like properties by altered regulation of cell cycle checkpoint and DNA repair network signaling. PLoS One 2014;9:e107142.

[19] L. Roy, S.J. Samyesudhas, M. Carrasco, et al., ARID3B increases ovarian tumor burden and is associated with a cancer stem cell gene signature, Oncotarget 5 (2014) 8355–8366.

[20] C. Samardzija, R.B. Luwor, M. Volchek, M.A. Quinn, J.K. Findlay, N. Ahmed, A critical role of Oct4A in mediating metastasis and disease-free survival in a mouse model of ovarian cancer, Mol. Cancer 14 (2015) 152.

[21] B. Davidson, CD24 is highly useful in differentiating high-grade serous carcinoma from benign and malignant mesothelial cells, Hum. Pathol. 58 (2016) 123–127.

[22] T.E. Hetland, E. Hellesylt, V.A. Flørenes, C. Tropé, B. Davidson, J. Kærn, Class III β-tubulin expression in advanced-stage serous ovarian carcinoma effusions is associated with poor survival and primary chemoresistance, Hum. Pathol. 42 (2011) 1019–1026.

[23] Siu MK, Wong ES, Kong DS, et al. Stem cell transcription factor NANOG controls cell migration and invasion via dysregulation of E-cadherin and FoxJ1 and contributes to adverse clinical outcome in ovarian cancers. Oncogene 2013;32:3500–9.

[24] C.M. Yamamoto, M.L. Oakes, T. Murakami, M.G. Muto, R.S. Berkowitz, S.W. Ng, Comparison of benign peritoneal fluid- and ovarian cancer ascites-derived extracellular vesicle RNA biomarkers, J. Ovarian Res. 11 (2018) 20.

[25] Raspaglio G, Petrillo M, Martinelli E, et al. Sox9 and Hif-2α regulate TUBB3 gene expression and affect ovarian cancer aggressiveness. Gene 2014;542:173–81.

[26] Bareiss PM, Paczulla A, Wang H, et al. SOX2 expression associates with stem cell state in human ovarian carcinoma. Cancer Res. 2013;73:5544–55.

[27] Belotte J, Fletcher NM, Alexis M, et al. Sox2 gene amplification significantly impacts overall survival in serous epithelial ovarian cancer. Reprod. Sci. 2015;22:38–46.

[28] J. Zhang, D.Y. Chang, I. Mercado-Uribe, J. Liu, Sex-determining region Y-box 2 expression predicts poor prognosis in human ovarian carcinoma, Hum. Pathol. 43 (2012) 1405–1412.

[29] Y. Wen, Y. Hou, Z. Huang, J. Cai, Z. Wang, SOX2 is required to maintain cancer stem cells in ovarian cancer, Cancer Sci. 108 (2017) 719–731.

[30] Pham DL, Scheble V, Bareiss P, et al. SOX2 expression and prognostic significance in ovarian carcinoma. Int. J. Gynecol. Pathol. 2013;32:358–67.

[31] Hellner K, Miranda F, Fotso Chedom D, et al. Premalignant SOX2 overexpression in the fallopian tubes of ovarian cancer patients: discovery and validation studies. EBioMedicine 2016;10:137–49.