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2 **SOX2 and SOX9 are markers of clinically aggressive disease in metastatic**

3 **high-grade serous carcinoma**

4 **Running title:** Stem cell markers in high-grade serous carcinoma

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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*.

Keywords: cancer stem cells; high-grade serous carcinoma; effusion; exosomes; chemotherapy response; survival

58 ## Introduction

59 Ovarian cancer, consisting mainly of ovarian carcinoma (OC), is the gynecologic malignancy
60 with the highest case to fatality ratio, mainly due to diagnosis at advanced stage (FIGO stage
61 III/IV) [1]. Chemoresistance, either intrinsic or acquired in the course of disease progression, is
62 an additional factor contributing to this poor outcome [2]. OC, and particularly its most common
63 histologic type, high-grade serous carcinoma (HGSC), has strong predilection to metastasis
64 within the abdominal cavity, characteristically forming both solid lesions and malignant ascites
65 [3]. OC cells in ascites constitute a chemoresistant cell population with an important role in
66 promoting tumor progression and fatal outcome in this cancer [2,4].
67
68 Cancer stem cells (CSC) represent a small population of cells during the initial tumor growth.
69 Chemotherapy will often eradicate the majority of tumor cells, but is ineffective in eliminating
70 CSC which, with time, proliferate and are the origin of disease recurrence. Several postulated
71 CSC markers have been identified in OC, including surface markers, such as CD24, CD44,
72 CD117 and CD133, and the intracellular cytoplasmic and/or nuclear proteins aldehyde
73 dehydrogenase isoform 1A1 (ALDH1A1), OCT4, Nanog, SOX2, Notch-1, nestin and others. The
74 presence of a side population identified by flow cytometry has been applied as an additional
75 criterion [5,6]. Several of these markers have been identified in OC CSC in ascites in
76 experimental models and/or patient material [7-20]. However, the clinical relevance of these
77 markers has not been assessed in large series of patients with OC effusions. Two recent studies
78 by a member of our group have failed to identify such role for CD24 and nestin in this anatomic
79 compartment [21,22].
80
81 The present study assessed the clinical and biological role of the CSC markers in HGSC.

82 **<u>Materials and methods</u>**

83 **Cell Lines and Reagents**

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

92

93 **Patients and specimens**

94 Specimens were submitted for routine diagnostic purposes to the Department of Pathology at the
95 Norwegian Radium Hospital during the period of 1998-2008. HGSC specimens and clinical data
96 were obtained from the Department of Gynecologic Oncology, Norwegian Radium Hospital. As
97 the fallopian tubes have not been adequately assessed in this cohort, tumors in the ovary are
98 specified as such without reference to primary site.
99 Tumors were diagnosed by an experienced gyn-pathologist and cytopathologist (BD). The
100 diagnosis of HGSC was made based on the combination of morphology (obvious nuclear atypia
101 and the presence of multiple mitoses) and the presence of aberrant (diffusely positive or entirely
102 negative) p53 immunostaining. Frozen sections from all solid tumors were reviewed by the same
103 author, and only specimens with tumor cell population >50% and minimal or no necrosis were
104 included in this study.

105 HGSC effusions analyzed using quantitative real-time reverse-transcription polymerase chain
106 reaction (qRT-PCR) consisted of 84 effusions (67 peritoneal, 17 pleural) from 84 patients. Forty-
107 one effusions were tapped at diagnosis and were chemo-naive and 42 were tapped after exposure
108 to chemotherapy. Chemotherapy status was unknown for 1 specimen. The 42 post-chemotherapy
109 specimens included 37 effusions tapped at disease recurrence, 4 effusions sampled in the primary
110 disease setting after administration of neoadjuvant chemotherapy and 3 effusions from patients
111 who only received chemotherapy.
112 Additionally, 50 solid lesions, including 30 ovarian resections and 20 solid metastases, the
113 majority omental, were analyzed for comparative purposes. The majority of specimens were not
114 patient-matched. However, patient-matched ovarian tumor and solid metastasis were available
115 from 3 patients and 2 metastases from the same patient in an additional case. Clinicopathologic
116 data are presented in **Table 1**.
117 Effusions were centrifuged immediately after tapping, and cell pellets were frozen at -70°C in
118 equal amounts of RPMI 1640 medium (GIBCO-Invitrogen, Carlsbad, CA) containing 50% fetal
119 calf serum (PAA Laboratories GmbH, Pasching, Austria) and 20% dimethylsulfoxide (Merck
120 KGaA, Darmstadt, Germany). Surgical specimens were frozen at -70°C without any treatment.
121 Additionally, 80 effusion supernatants (59 peritoneal, 21 pleural) collected in the years 1998-
122 2003 from which exosomes were isolated were frozen at -70°C without any treatment.
123 Informed consent was obtained according to national and institutional guidelines. Study approval
124 was given by the Regional Committee for Medical Research Ethics in Norway.
125
126 **qRT-PCR**
127 cDNA was transcribed of 500ng total RNA. qRT-PCR was carried out using the KAPA SYBR
128 FAST qPCR kit (Kapa Biosystems, Wilmington MA) according to the manufacturer's protocol.

129 Specificity was confirmed by appropriate melting curves. mRNA levels were established by
130 calculating the target molecule: reference gene (*RPLP0*) ratio. Primer sequences are listed in
131 **Table 2**.
132

133 **Immunohistochemistry (IHC)**

134 Formalin-fixed, paraffin-embedded sections from the 52 of the 84 HGSC effusions analyzed
135 using qRT-PCR were immunohistochemically analyzed for SOX2, SOX9, OCT3/4, and Nanog
136 expression using the Dako EnVision™ FLEX (OCT3/4 and Nanog) or FLEX+ (SOX2 and
137 SOX9) System (Agilent/Dako, Glostrup, Denmark). Following deparaffinization, sections were
138 incubated with a 0.3% hydrogen peroxide (H2O2) solution for 5 minutes to block endogenous
139 tissue peroxidase activity. Sections were then incubated with the relevant antibody. Antibody
140 details and staining were as follows:
141 SOX2 mouse monoclonal antibody (clone 245610; cat#MAB2018; R&D systems, Minneapolis
142 MN): 1:200 dilution, antigen retrieval in citrate buffer (pH 6).
143 SOX9 mouse monoclonal antibody (clone 3C10; cat#ab76997; Abcam, Cambridge UK): 1:5000
144 dilution, antigen retrieval in citrate buffer (pH 6).
145 OCT3/4 goat polyclonal antibody (cat# AF1759; R&D systems): 1:400 dilution, antigen retrieval
146 in citrate buffer (pH 6).
147 Nanog goat polyclonal antibody (cat# AF1997; R&D systems): 1:200 dilution, antigen retrieval
148 in citrate buffer (pH 6).
149 Sections were thereafter treated with EnVision™ Flex+ mouse or goat linker (15 min) and
150 EnVision™ Flex/HRP enzyme (30 min), stained for 10 min with 3,3 diaminobenzidine
151 tetrahydrochloride (DAB), counterstained with hematoxylin, dehydrated and mounted in Richard-
152 Allan Scientific Cyto seal XYL (Thermo Fisher Scientific). Positive controls consisted of normal

153 testis. Negative controls were stained with nonrelevant antibody of the same isotype for
154 monoclonal antibodies and normal goat serum for polyclonal antibodies.
155

156 **Western blotting (WB)**

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

170 GAPDH was used as loading control for proteins originating from cells and CD63 was used as a
171 loading control for proteins originating from exosomes. Proteins were detected using EZ-ECL
172 Chemiluminescence detection kit for HRP (Biological Industries). Densitometer analysis of films
173 was performed using a computerized image analysis program (Image-J, NIH, Bethesda MD).
174

175 **CRISPR/Cas9 knockout (KO)**

176 The vector was digested by the BBSI restriction enzyme (New England Biolabs, Ipswich MA).
177 Extraction and purification of digested plasmid from agarose gel of PCR products was done with
178 Nucleospin® Gel and PCR Clean-up Kit (Macherey-Nagel, Düren, Germany). Ligation was
179 performed with T4 DNA ligase (New England Biolabs). Specific single guide RNA (sgRNA)
180 inserts were designed with the help of Zhang Lab scoring (http://crispr.mit.edu/) and were
181 targeted at the beginning of the 5' of the *NANOG* gene:
182 Forward: CACCTGTCGCAAAAAAGGAAGACA
183 Reverse: AAACTGTCTTCCTTTTTTGCGACA
184 The insert selected targeted Exon 2 of Nanog in order to stop early translation. The inserts were 5'
185 phosphorylated and annealed on a ramp between 95-25°C. The plasmid (pSpCas9 BB -2A- GFP,
186 PX458 plasmid # 48138 vector) was a generous gift from the lab of Prof. Yehudit Bergman at the
187 Hebrew University of Jerusalem. The plasmid was digested by the BBSI restriction enzyme.
188 Ligation was performed with T4 ligase. The plasmids were then transformed to competent DHα5
189 E. Coli bacteria. Plasmids were extracted with a commercial kit, GeneJet Plasmid Miniprep
190 (Fermentas Life Sciences/Thermo Fisher, Waltham MA) and sent to sequencing in order to
191 confirm the insertion of the sgRNA. Thereafter, the plasmids were transfected into OVCAR3
192 cells with Lipofectamine 3000 (Invitrogen, Carlsbad CA). Sorting by FACS Aria II (Ein Karem,
193 Hebrew University) was performed and the GFP positive sorted cells were seeded as single cell
194 colonies, which resulted as single cell clone KO cells, subsequently analyzed for KO by WB
195 analysis.
196

197 **siRNA**

198 SOX2 and SOX9 were silenced using the following siRNAs:
199 SOX2: mix of SASI_Hs01_00050572, SASI_Hs01_00050573, SASI_Hs01_00050580

200 SOX9: EHU021061

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

202

203 **Exosome extraction and quantitation**

204 Clinical specimens: Exosomes were extracted from 250µl of effusion fluid according to the user's

205 manual of ExoQuick-TC (System Biosciences, Mountain View CA) and quantified with the

206 Bradford assay. Approximately 27 and 100 whole exosomes were tested for protein and mRNA,

207 respectively.

208 Cell line: $1x10^7$ cells were seeded and cultured in serum-free medium, BSA 0.1% for 24 hours.

209 Conditioned medium was filtered with a 0.1µM PVDF (Merck Millipore, Tullagreen, Ireland)

210 filter and then concentrated with 3000 MWCO vivaspin 20 (Santorius, Göttingen, Germany).

211 Exosomes were extracted from the supernatant according to the ExoQuick-TC manual. The

212 resulting pellet containing exosomes was re-suspended in 100µl PBS and analyzed for protein

213 concentration by the Bradford assay.

214

215 **Scratch Assay**

216 OVCAR3, Nanog KO-C and Nanog KO-E cells (400,000) were seeded in 6-well plates. They

217 were treated with 10µg OVCAR3 exosomes and incubated for 24 hours in DMEM serum-free

218 medium, BSA 0.1%. Prior to assay the cells were washed with PBS and replaced with DMEM

219 BSA 0.1% without exosomes. Each well was scratched twice with a sterile tip and imaged at t=0,

220 t=6 and t=24 hours. The closure of the wound was analyzed by T-scratch software51.

221

222 **Proliferation Assay**

223 OVCAR8, OVCAR3, Nanog KO-C and Nanog KO-E cells (400,000) were seeded in 6-well
224 plates and were treated with 10μg OVCAR3, KO-C and KO-E exosomes for 24 hours in serum-
225 free medium, BSA 0.1%. At 24 hours, cells were treated with 0.5mg/mL 3-(4,5-dimethylthiazol-
226 2-yl)-2,5-diphenyltetrazolium bromide (MTT; EMD) for 30 minutes. Cells were then lysed with
227 DMSO (Merck) and the absorbance of the solution was read at 560nm using Multiscan RC
228 (Thermo Fisher).
229

230 **Invasion Assay-Boyden Chamber**

231 OVCAR8, OVCAR3, Nanog KO-C and Nanog KO-E cells (400,000) were seeded in 6-well
232 plates and were treated with 10μg OVCAR3, KO-C and KO-E exosomes for 24 hours in serum-
233 free medium, BSA 0.1%. Cells were than seeded on 8.0 μM PVDF filters (GE Whatman, Little
234 Chalfont, Buckinghamshire, UK) coated with 25μg Matrigel in Boyden chambers. On the
235 opposite side, a chemoattractant (conditioned medium from the 3T3 fibroblast cell line) was
236 placed. After 6 hours of incubation in optimal conditions, filters were removed and the presence
237 of invading cells was determined by staining and counted.
238

239 **Zymography**

240 OVCAR8 cells (400,000) were seeded in 6-well plates. They were treated with 10μg OVCAR3,
241 KO-C and KO-E exosomes and incubated for 24 hours in serum-free medium, BSA 0.1%.
242 Samples from the supernatants were collected after 24 hours and were analyzed for collagenolytic
243 activity, determined on 1mg gelatin/ml , 10% SDS-PAGE gel. Bands were analyzed by ImageJ
244 software.
245

246 **Statistical analysis**

247 Statistical analysis was performed applying the SPSS-PC package (Version 25, Chicago IL).
248 Probability of <0.05 was considered statistically significant. Comparative analysis of CSC
249 marker expression in effusions, ovarian tumors and solid metastases was performed using the
250 Kruskal-Wallis H test. Analysis of the association between expression levels of these molecules
251 in HGSC effusions and clinicopathologic parameters was executed using the Mann-Whitney U
252 (for 2 groups) or Kruskal-Wallis H (for 3 groups) test. For this analysis, as well as for survival
253 analysis, clinicopathologic parameters were grouped as follows: age: ≤60 vs. >60 years; effusion
254 site: peritoneal vs. pleural; FIGO stage: III vs. IV; chemotherapy status: pre- vs. post-
255 chemotherapy specimens; residual disease (RD): 0 cm vs. ≤1 cm vs. >1 cm; response to
256 chemotherapy: complete response vs. partial response/stable disease/progressive disease.
257 Progression-free survival (PFS) and overall survival (OS) were calculated from the date of the
258 last chemotherapy treatment/diagnosis to the date of recurrence/death or last follow-up,
259 respectively. Univariate survival analyses of PFS and OS were executed using the Kaplan-Meier
260 method and log-rank test. Platinum resistance was defined as PFS≤6 months according to
261 guidelines published by the Gynecologic Oncology Group and progressive disease or recurrence
262 was evaluated by RECIST criteria. Multivariate survival analysis was performed using the Cox
263 regression model (Enter function).

264 Analysis KO metastatic assays were performed using a two-tailed student T-test.

265

266 ## Results

267 ### *SOX9* and *OCT4* are differentially expressed at different anatomic sites in HGSC

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

274

275 ### Association with clinicopathologic parameters and survival

276 The clinical relevance of the studied molecules was analyzed in the effusion cohort, which
277 included the largest number of patients. *OCT4* mRNA levels were significantly higher in pleural
278 effusions compared to peritoneal specimens (p=0.03). Higher *SOX2* and *SOX9* expression was
279 significantly related to intrinsic chemoresistance (PFS≤6 months; p=0.009 and p=0.02,
280 respectively), and showed a trend towards higher expression in patients with poor chemoresponse
281 to first-line chemotherapy (p=0.077 and p=0.088, respectively).
282 CSC marker expression was unrelated to previous exposure to chemotherapy, patient age, FIGO
283 stage or RD volume (p>0.05; data not shown).

284

285 The follow-up period for the 84 patients with HGSC effusions ranged from 1 to 179 months
286 (mean = 37 months, median = 26 months). PFS ranged from 0 to 81 months (mean = 10 months,
287 median = 6 months). At the last follow-up, 78 patients were dead of disease, 3 were alive with
288 disease and 1 was with no evidence of disease. One patient died of treatment complications and 1

289 was lost to follow-up. The association between CSC marker expression, as well as clinical
290 parameters (age, FIGO stage and RD volume), and survival was analyzed.
291 In univariate survival analysis of all cases, higher *SOX9* levels were associated with shorter OS
292 (p=0.04; **Figure 1-A**). None of the other CSC markers or clinical parameters was significantly
293 associated with survival. Parameters with p-value <0.2, including *NANOG* levels (p=0.172) and
294 patient age (p=0.109), were entered into the Cox multivariate analysis with *SOX9*. Higher *SOX9*
295 levels (p=0.049) and older age (p=0.04) were independent prognostic markers in this analysis.
296 In separate survival analysis for patients with pre-chemotherapy effusions tapped at diagnosis and
297 patients with post-chemotherapy specimens, a trend for worse OS was observed for *SOX9* levels
298 in pre-chemotherapy effusions (p=0.053), with no other findings (data not shown).
299

300 **SOX proteins and OCT3/4 proteins, but not Nanog, are expressed in HGSC effusions**

301 IHC analysis of HGSC effusions showed universal expression of SOX9 protein, particularly at
302 the tumor cell nuclei, with more variable SOX2 and OCT3/4 protein expression, while Nanog
303 was uniformly absent in the 52 studied specimens (**Figure 2; Table 3**). Higher cytoplasmic
304 SOX9 expression was significantly related to intrinsic chemoresistance (PFS≤6 months;
305 p=0.015). Higher SOX2 protein expression was associated with shorter OS in univariate analysis
306 (p=0.049). Multivariate analysis was not performed as none of the clinical parameters was
307 associated with OS (P>0.05; data not shown).
308 In view of the unexpected absence of Nanog from HGSC cells we investigated the possibility that
309 this molecule was localized to an extracellular compartment in HGSC effusions. In agreement
310 with this hypothesis, *NANOG* mRNA was found in exosomes in 71/80 (89%) specimens, and
311 Nanog protein was found in all specimens in analysis of 24 effusion exosome preparations by
312 WB (**Figure 3-A**). *SOX2*, *OCT4* and *LIN28* mRNA was not found in HGSC exosomes. *NANOG*

313 mRNA levels in exosomes were unrelated to clinicopathologic parameters or to survival (p>0.05;
314 data not shown).
315

316 **CRISPR Cas9 KO in OVCAR3 cells**

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

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

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

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

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

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

337 **Discussion**

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

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

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

356 Hu and co-workers detected Nanog in OC ascites. However, their study included a single
357 specimen and cells were cultured rather than analyzed in their native state [8]. Wintzell et al.
358 identified this protein in OC ascites, with higher expression in cells growing in spheroids
359 compared to those growing as monolayer [13], and our data are consequently not in agreement
360 with this study. Nanog was detected in rare cells in ascites in the study by Di et al., but the

361 number of specimens analyzed for this marker is unclear [15]. Recently, Yamamoto and co-
362 workers reported that Nanog levels are significantly higher in extracellular vesicles from HGSC
363 ascites compared to benign peritoneal fluid [24], a finding well in agreement with our observation
364 that Nanog is localized to exosomes in these specimens. Data generated from our silencing
365 experiments suggests that Nanog in exosomes regulates invasion, migration and possibly protease
366 activation, in HGSC, while having no effect on proliferation.
367
368 Analysis of the clinical relevance of CSC marker expression in HGSC effusions identified SOX2
369 and SOX9 as candidate markers of poor chemoresponse and shorter survival, and silencing of
370 these 2 genes in OC cells suppressed invasion, migration and proteolytic activity. Data regarding
371 the clinical role of SOX9 in OC is limited to date. However, our findings are in agreement with
372 the observed association between expression of this marker and poor outcome in primary
373 carcinomas of different histotypes, the majority of serous type [25]. Of note, in the latter study,
374 SOX9 was shown to interact with the promoter of *TUBB3*, the gene encoding for class III β-
375 tubulin, a protein whose expression in OC effusions we reported to be associated with
376 chemoresistance and poor survival [26].
377 Data supporting the clinical role of SOX2 in OC are available from several studies. In the study
378 by Bareiss et al., SOX2 expression increased the expression of other CSC markers and tumor
379 formation of spheres in OC cells, and promoted tumorogenicity *in vivo*. SOX2 did not affect
380 proliferation, but mediated apoptosis resistance following chemotherapy and TRAIL treatment
381 [27]. *SOX2* amplification was associated with poor survival in analysis of the TCGA dataset [28].
382 Zhang et al. analyzed 540 carcinomas, the majority HGSC, for SOX2 protein expression using
383 IHC. SOX2 was expressed in 79 tumors (15%), most often in HGSC and carcinosarcoma, and
384 was associated with shorter disease-free survival in univariate, though not multivariate survival

385 analysis [29]. In the study by Wen et al., SOX2 was overexpressed in SKOV3 spheroids
386 compared to monolayers. SOX2 knockdown in SKOV3 and HO8910 spheroids reduced spheroid
387 formation, proliferation, migration, resistance to cisplatin, tumorigenicity in mice, and the
388 expression of CSC and EMT-related genes, whereas SOX2 overexpression had the opposite
389 effects. SOX2 protein expression by IHC was associated with chemoresistance and poor OS and
390 PFS in analysis of 53 tumors specified as type I (n=12) or type II (n=41) [30].
391 In a single study suggesting association with better outcome, SOX2 protein expression by IHC
392 was associated with longer disease-free survival for patients with stage II-IV OC, with no such
393 role for SOX2 amplification [31].
394 Of note, SOX2 mutation was found in fallopian tube epithelium with normal morphology in a
395 patient with HGSC, and overexpression of SOX2 protein was commonly observed in fallopian
396 tube epithelium with normal morphology in patients with HGSC and in *BRCA1*/*BRCA2* mutation
397 carriers who underwent prophylactic salpingo-oophorectomy, while being much less common in
398 fallopian tubes from patients with benign conditions, suggesting that expression of this CSC
399 marker may be an early event in the development of HGSC [32].
400
401 In conclusion, our data suggest an association between SOX2 and SOX9 expression at the mRNA
402 and protein level and aggressive clinical behavior in HGSC metastases to serous effusions.
403 Nanog may mediate disease progression via signals generated from exosomes.

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406

407 **Conflict of interest statement:** We have no conflict of interest

408

409 **Author contributions:**

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

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

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

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

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

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497

498 **Table and Figure legends**

499 **Table 1: Clinicopathologic parameters for HGSC patients with effusion (n=84), ovarian**
500 **tumor (n=30) and solid metastasis (n=19)**

501

502 **Table 2: Primer sequences**

503

504 **Table 3: IHC results in 52 HGSC effusions**

505

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

516

517 **Figure 2: SOX2, SOX9 and OCT3/4 protein expression in HGSC effusions**
518 (A-B): Two effusions with SOX2 expression in HGSC cells. Expression is nuclear in (A),
519 predominantly cytoplasmic (few positive nuclei) in (B)
520 (C-D): Two effusions with SOX9 expression in HGSC cells. Expression is nuclear in (C),
521 combined nuclear and cytoplasmic in (D)

522 (E): Nuclear OCT3/4 nuclear expression. Staining is seen in nucleoli and chromatin clusters.

523 (F): Negative Nanog staining.

524

525 **Figure 3: Nanog in HGSC exosomes and cell lines**

526 **3-A:** Nanog protein expression in effusion-derived exosomes**.**

527 **3-B:** Nanog protein expression in OVCAR3 cell line and its downregulation in the two KO-Cell

528 lines created; $p<0.01$ for both cell lines.

529 **3-C:** Nanog protein expression in OVCAR3 exosomes and its downregulation in the two KO-

530 exosomes created; $p<0.01$ for both cell lines.

531 **3-D-3F:** Scratch Assay.

532 3-D: Graph depicting % of wound closure in KO-E cell lines treated with OVCAR3 exosomes

533 and untreated cells at 6 and 24 hours; $p<0.01$.

534 3-E: Graph depicting % wound closure in KO-C cell lines treated with OVCAR3 exosomes and

535 untreated cells at 6 and 24 hours; $p<0.05$.

536 3-F: Graph depicting % of wound closure in OVCAR3, KO-C and KO-E cells without any

537 treatment at 6 and 24 hours.

538 **3-G:** Invasion Assay. Treatment with Nanog KO-exosomes reduces invasion ($p<0.05$).

539 **3-H:** MTT proliferation assay of OVCAR8, ES2, OVCAR3, KO-C and KO-E cell lines treated

540 with OVCAR3, KO-C and KO-Exosomes. Proliferation is not significantly affected ($p>0.05$).

541 **3-I:** MMP9 activity. Nanog KO induces a slight decrease MMP9 activity, evidenced as reduced

542 ability of tumor cells to degrade gelatin.

543 **3-J:** Invasion Assay. SOX2 and SOX9 siRNA significantly reduces invasion ($p<0.05$).

544 **3-K:** Wound healing assay. SOX2 and SOX9 siRNA significantly reduces motility ($p<0.05$).

545 **3-L:** MMP2 activity. SOX2 and SOX9 siRNA significantly reduces gelatinolytic activity

546 ($p<0.05$).

547

548 **Table 1: Clinicopathologic parameters for HGSC patients with effusion (n=84), ovarian**

549 **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]	11-43800 (800)	178-28000 (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

550

551 Abbreviations: NA = not available; CR = complete response; PR = partial response; SD = stable

552 disease; PD = progressive disease

553 [a] Three patients with both ovarian tumor and solid metastasis are represented in both columns.

554 One patient with 2 solid metastases is listed only once in the metastasis column.

555 [b] Values for 53 patients who received surgery a upfront treatment were as follows: 0 cm: 6

556 patients; ≤1 cm: 23 patients; >1 cm: 23 patients; unknown: 1 patient.

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

558 patients with solid metastasis

559 **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'

560

561 **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

562

563 [a] One missing case

564

565

566 **Highlights**

567 • Higher SOX2 and SOX9 expression in HGSC effusions is associated with primary
568 chemoresistance and shorter survival
569 • Nanog is secreted from HGSC cells into exosomes in effusion supernatants.
570 • Nanog knockout *in vitro* suppresses reduced migration, invasion and MMP9 activity.
571