Dear Editor

We are thankful to you and the reviewers for providing us with comprehensive reviews of our work. We performed major and minor revisions of our manuscript based on their comments and suggestions in order to improve it. Here, we provide a rebuttal of the changes we made. We shall be grateful for any further comments and suggestions.

Sincerely yours,

Nataša Kenda Šuster

Rebuttal/2019-04-23

Reviewers' comments:

Reviewer #1:

Comment: "This is an elegant review paper to summarize the current updates of cancer stem cells (CSC) derived from ovarian, and to discuss future perspectives in the field of ovarian cancer therapy using CSC. It will be much appreciated if the authors could add the following contents in this manuscript.

1) The CSC derived vesicles and the role for tumor progression, etc.

Answer: The following content with corresponding references was added to manuscript to page 11-12, chapter 5: Ovarian cancer stem cell microenvironment

"Beside soluble factors and proteins secreted by ovarian cancer cells, the role of extracellular vesicles in the formation of pre-metastatic niche and metastatic colonization has been investigated. Exosomes play an important role in intercellular signaling and in transportation of genetic information. They are coordinators between tumor cells, stromal cells and the extracellular matrix through the shuttling of different lipids, proteins, double-stranded DNAs, RNAs, non-transcribed RNAs, and microRNAs. Ovarian cancer derived exosomes were reported to transfer CD44 into mesothelial cells, upregulating matrix metalloproteinase 9 (MMP9) that facilitates cancer cell nesting and invasion. Exosomes in epithelial ovarian cancer have also promising therapeutic potential as they are related to immune system, tumor microenvironment and tumor angiogenesis. Extracellular vesicles have created new perspective on diagnosis, prognosis, treatment, and drug resistance in

ovarian cancer, however, knowledgebase has so far been limited, so further research is needed."

2) Interactions between CSC and the local tissues and cells.

Answer: The following content with corresponding references was added to manuscript to page 10-12, chapter 5: Ovarian cancer stem cell microenvironment

»Defining the niche that supports ovarian CSCs must consider the clinical course of the disease. The evolution of ovarian cancer, its origin in the ovarian surface epithelium or in the distal part of fallopian tube, its progression, and especially its peritoneal dissemination, indicates the existence of multiple types of niches. Within primary tumors, multiple stromal cell types are involved in the formation of a pro-tumorigenic microenvironment. Tumor cells release several soluble factors and proteins that mobilize the population of tumor cells to settle in distant organs and tissues. 3D cultures that displayed the early dissemination of ovarian cancer into peritoneal mesothelium reveled, that cancer cells induce mesothelial cells to synthesize fibronectin via secretion of transforming growth factor beta-1 (TGFß1). Ascites, frequently accompanying advanced disease, represents a unique type of ovarian CSC microenvironment. Interleukin 6 (IL-6), being elevated in ascites, triggers the JAK /STAT3 signaling pathway, which plays an important role in ovarian CSC function. The Wnt/beta-catenin pathway is another pathway involved in communication between ovarian cancer cells and ascites, thus its inhibition presents potential therapeutic target for ovarian cancer. The adipose tissue, especially omentum, provides another microenvironment, optimal for ovarian cancer lesions. Omental adipocytes enable nesting, invasion, and migration of ovarian cancer cells, and provide energy for rapid tumor growth.«

3) Please cite more recent references to add the value of this manuscript

Answer: The following references from 52-62 were added to manuscript to chapter 5: Ovarian cancer stem cell microenvironment

52 Peinado H, Zhang H, Matei IR, Costa-Silva B, Hoshino A, Rodrigues G, Psaila B, Kaplan RN, Bromberg JF, Kang Y, Bissell MJ, Cox TR, Giaccia AJ, Erler JT, Hiratsuka

S, Ghajar CM, Lyden D. Pre-metastatic niches: Organ-specific homes for metastases. Nat Rev Cancer 2017;17:302-317 [PMID: 28303905 DOI: 10.1038/nrc.2017.6]
53 Robado de Lope L, Alcibar OL, Amor Lopez A, Hergueta-Redondo M, Peinado H. Tumour-adipose tissue crosstalk: Fuelling tumour metastasis by extracellular vesicles. 2018;373 [PMID: 29158314 DOI: 10.1098/rstb.2016.0485]
54 Kenny HA, Chiang CY, White EA, Schryver EM, Habis M, Romero IL, Ladanyi A, Penicka CV, George J, Matlin K, Montag A, Wroblewski K, Yamada SD, Mazar AP, Bowtell D, Lengyel E. Mesothelial cells promote early ovarian cancer metastasis through fibronectin secretion. J Clin Invest 2014;124:4614-4628 [PMID: 25202979 DOI: 10.1172/jci74778]
55 Abubaker K, Luwor RB, Zhu H, McNally O, Quinn MA, Burns CJ, Thompson EW, Findlay JK, Ahmed N. 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 2014;14:317 [PMID: 24886434 DOI: 10.1186/1471-2407-14-317]
56 Bharti R, Dey G, Mandal M. Cancer development, chemoresistance, epithelial to mesenchymal transition and stem cells: A snapshot of il-6 mediated involvement. Cancer Lett 2016;375:51-61 [PMID: 26945971 DOI: 10.1016/j.canlet.2016.02.048]
57 Kim S, Gwak H, Kim HS, Kim B, Dhanasekaran DN, Song YS. Malignant ascites enhances migratory and invasive properties of ovarian cancer cells with membrane bound il-6r in vitro. Oncotarget 2016;7:83148-83159 [PMID: 27825119 DOI: 10.18632/oncotarget.13074]
58 Arend RC, Londono-Joshi AI, Samant RS, Li Y, Conner M, Hidalgo B, Alvarez RD, Landen CN, Straughn JM, Buchsbaum DJ. Inhibition of wnt/beta-catenin pathway by niclosamide: A therapeutic target for ovarian cancer. Gynecol Oncol 2014;134:112-120 [PMID: 24736023 DOI: 10.1016/j.ygyno.2014.04.005]
59 Nieman KM, Kenny HA, Penicka CV, Ladanyi A, Buell-Gutbrod R, Zillhardt MR, Romero IL, Carey MS, Mills GB, Hotamisligil GS, Yamada SD, Peter ME, Gwin K, Lengyel E. Adipocytes promote ovarian cancer metastasis and provide energy for rapid tumor growth. Nat Med 2011;17:1498-1503 [PMID: 22037646 DOI: 10.1038/nm.2492]
60 Cheng L, Wu S, Zhang K, Qing Y, Xu T. A comprehensive overview of exosomes in ovarian cancer: Emerging biomarkers and therapeutic strategies. 2017;10:73 [PMID: 29100532 DOI: 10.1186/s13048-017-0368-6]
61 Nakamura K, Sawada K, Kinose Y, Yoshimura A, Toda A, Nakatsuka E, Hashimoto K, Mabuchi S, Morishige KI, Kurachi H, Lengyel E, Kimura T. Exosomes promote ovarian cancer cell invasion through transfer of cd44 to peritoneal mesothelial cells. Mol Cancer Res 2017;15:78-92 [PMID: 27758876 DOI: 10.1158/1541-7786.mcr-16-0191]
62 Li X, Wang X. The emerging roles and therapeutic potential of exosomes in epithelial ovarian cancer. Mol Cancer 2017;16:92 [PMID: 28506269 DOI: 10.1186/s12943-017-0659-y]

Reviewer #2:

Comment: In this manuscript, the authors discussed the current research on CSCs in ovarian cancer, focusing on CSCs development and their role in tumor formation, progression and recurrence after, allegedly, successful treatment. The topic is is within the range of publication of the journal. The manuscript could provide some new ideas and new progress on this research direction. However, there're still several issues which should be addressed.

1.) The biomarks of the stem cells in ovarian cancer is one important question. So, the author should add relevant content and fully discuss it. Moreover, the authors should tell the readers how to study it and discuss the technique.

Answer: The following content with corresponding references was added to manuscript to page 12-13, chapter 6: Ovarian cancer stem cell identification: the biomarkers

»Identification of CSCs relies on the presence of markers. In ovarian cancer many markers are used to confirm the presence of CSCs (Table 1). Isolated CSCs can then be tested for stemness in vitro through spheroid forming assay, and in vivo with limiting dilution assays to examine the tumorigenicity of the sample on the animal model.

There are multiple surface biomarkers used to identify CSCs in ovarian cancer. CD117 was demonstrated to be the first cell surface marker for the ovarian CSCs. Its expression correlates with tumor formation, chemoresistance, and poor prognosis of disease. CD 133 is one of the most commonly reported ovarian CSC surface markers. It is associated with a number of stem characteristics, like tumor formation, disease progression, chemoresistance, and poor prognosis. It was also studied as a target for a cancer target therapy. Other common CSC surface markers are CD24, CD44, EpCAM and ROR1. CD24 is associated with tumor formation, metastasis, poor prognosis, chemoresistance, and recurrence of disease. Similar characteristics are correlated with CD 44. Surface markers can be used alone or in combination with other ovarian CSC markers.

In addition to cell surface markers, the enzyme aldehyde dehydrogenase 1 (ALDH1) is used to identify CSCs in ovarian cancer. Several studies correlate ALDH1 expression with cell proliferation, migration promotion, poor survival, and

chemoresistance. Conversely, inhibition of ALDH1A1 in a mouse model sensitized the tumors to treatment. The expression of ALDH1 alone or in combination with cell surface stem cell markers is an accepted method for CSC identification in ovarian cancer.

NANOG is a transcription factor that, along with transcription factors OCT4 and SOX2, plays a key role in pluripotency and self-renewal maintenance in undifferentiated embryonic stem cells. NANOG, OCT4 and SOX2 are commonly expressed also in ovarian CSCs. Their expression is associated with poor prognosis and chemoresistance. NANOG also regulates epithelial-mesenchymal transition. c-Myc is another key oncogenic transcription factor that participates in tumor pathogenesis. Its knockdown by let-7d increases ovarian cancer cell sensitivity to a genistein analog. Future studies on pluripotency factors expressed in ovarian CSCs will provide additional data on how cancer stemness is maintained.

Another way in which ovarian CSCs can be identified is by the ability to efflux DNA-binding dyes resulting in a side population using flow cytometry. For dye effluxion CSCs should express ATP binding cassette transporters such as MDR1/ABCB1 and ABCG2 that can efflux chemotherapeutic agents and so contribute to chemoresistance. »

2.)Heterogeneous nature of ovarian cancer is very important too. So, the relevant content should be added in.

Answer: The following content with corresponding references was added to manuscript to page 4, chapter 1: Ovarian cancer

"Ovarian cancer presents a heterogeneous group of tumors: epithelial, germ cell, and stromal cells tumors. Approximately 90% of ovarian cancer belong to the malignant epithelial tumor (carcinomas) group and, based on histopathology, immunohistochemistry, and molecular genetic analysis, five main types of carcinoma are currently known: high grade serous carcinoma, 70%; low grade serous carcinoma <5%; endometrioid carcinoma, 10%; clear cell carcinoma, 10%; and mucinous carcinoma 3%. Different epithelial malignancies have, in addition to their different origin and morphologies, different biological behaviour. Low grade serous

carcinoma arises from fallopian tube, endometrioid carcinoma, clear cell carcinoma, and seromucinous carcinoma arise from endometriosis, mucinous carcinoma arises from germ cells, and malignant Brenner tumor arises from transitional epithelium. All are slow-growing tumors which develop progressively from benign and borderline precursor lesions to malignancy. They are genetically stable, characterized by mutations in different genes: KRAS, BRAF, PTEN, ß-catenin, and others. High grade ovarian serous carcinoma, on the other hand, has a high level of genetic instability and is characterized by TP53 mutation, and loss of BRCA1 and BRCA2 function. It is fast-growing and highly aggressive neoplasm, with massive disease in the omentum and the mesentery, usually accompanied by ascites. There are two models considered, high grade ovarian serous carcinoma arising from the ovarian surface epithelium or from the fallopian tube. As both tissues are derived from the same embryologic origin, high grade ovarian serous carcinoma may arise from two different sites that undergo similar changes. Progenitor cells from different sites may respond similarly. However, BRCA deficiency and simultaneously presence of the intraepithelial carcinoma in the fallopian tube (serous tubal intraepithelial carcinoma) make fallopian tube model of high grade ovarian serous carcinoma origin more relevant."

Reviewer #3:

Comment: The authors present a concise, interesting and readable review discussing the stem cell compartment in ovarian cancer and how it can be targeted for treatment. Major comments :

1.) The authors should refer here to the ongoing discussion regarding the origin of ovarian cancer. Is it derived from the ovarian surface epithelium or from the fallopian tube (see e.g. Klotz DM and Wimberger P (2017) Arch Gynecol Obst 296: 1055-1062). This may determine where one should look for stem cells.

Answer: The following content with corresponding references was added to manuscript to page 4-5, chapter 1: Ovarian cancer

"High grade ovarian serous carcinoma, on the other hand, has a high level of genetic instability and is characterized by TP53 mutation, and loss of BRCA1 and BRCA2

function. It is fast-growing and highly aggressive neoplasm, with massive disease in the omentum and the mesentery, usually accompanied by ascites. There are two models considered, high grade ovarian serous carcinoma arising from the ovarian surface epithelium or from the fallopian tube. As both tissues are derived from the same embryologic origin, high grade ovarian serous carcinoma may arise from two different sites that undergo similar changes. Progenitor cells from different sites may respond similarly. However, BRCA deficiency and simultaneously presence of the intraepithelial carcinoma in the fallopian tube (serous tubal intraepithelial carcinoma) make fallopian tube model of high grade ovarian serous carcinoma origin more relevant."

"Treating disease in its advanced course is demanding and often unsuccessful, so defining the origin of ovarian cancer and performing suitable prophylactic surgery like oophorectomy or salpingectomy may save many lives. "

2.) Can the authors indicate whether the different stem cells compartments that are apparently present in ovarian cancer, have been analysed at the DNA level e.g. by whole genome sequencing. The presence of specific mutation / genomic aberration may help to identify the culprit cancer stem cell compartment.

Answer: The following content with corresponding references was added to manuscript to page 13-15, chapter 7: Ovarian cancer stem cells in the era of "omics" and gene expression profiling

Although CSCs can cause different processes such as tumor initiation, malignant proliferation, relapse and multi-drug resistance, the way to eliminate CSCs remains unknown. The modern molecular genetic methods enable to study the ovarian CSCs in a more detail, in terms of their gene expression profile (e.g., whole genome sequencing). An increasing number of studies try to elucidate whether the different stem cells compartments that are apparently present in ovarian cancer differ at the DNA level. These studies showed a niche-dependent gene expression profile of heterogeneous intratumoral populations of stem cells, which makes a task to target ovarian CSCs even more difficult.

It was found that TP53 is the most frequently mutated gene in high grade ovarian cancer. About 50% of these tumors showed defective homologous recombination due

to germline and somatic BRCA mutations, epigenetic inactivation of BRCA and abnormalities of DNA repair genes. Along this, somatic copy number alterations are frequent in these tumors, especially defective NOTCH, RAS/MEK, PI3K and FOXM1 pathway signaling. Some of them are associated with patients' prognosis. Other subtypes of ovarian cancer were characterized by a different mutational spectrum: low grade ovarian serous carcinoma has increased frequency of BRAF and RAS mutations, mucinous cancers have a mutation in ARID1A, PIK3CA, PTEN, CTNNB1 and in RAS genes. Some data also suggest that TERT C228T promoter mutations may have an important role in progression of adult granulosa cell tumors. Intensive research was focused on relation between the gene expression profile and ovarian CSCs. For identification of CSCs positivity for some markers, including CD133, CD44, CD117, CD24, EpCAM, LY6A, and ALDH1, was used.

An important task remains to elucidate the originating cancer stem cells (e.g., VSELs) and common characteristics of different populations of stem cells involved at different stages of ovarian cancer. Some studies compared the gene expression profile of ovarian CSCs in ovarian cancer of different grades, including advanced disease compared to normal ovarian surface epithelium, to identify the key pathways, and specific molecular signatures involved in the manifestation of ovarian cancer at different stages. Comparison of genome-wide expression profiles in ovarian CSCs revealed a mass of differentially expressed genes. Among these genes, NAB1 and NPIPL1 were commonly upregulated, whereas the genes PROS1, GREB1, KLF9 and MTUS1 were commonly downregulated, regardless of the stage of disease. These genes regulate the cellular components such as centrosome, plasma membrane receptors, and basal lamina, and may participate in biological processes such as cell cycle regulation, chemoresistance and stemness induction. Moreover, the gene co-expression extrapolation screening by the Connectivity Map revealed several small-molecule compounds (such as SC-560, disulfiram, thapsigargin, esculetin and cinchonine) with potential anti-ovarian CSCs properties targeting ovarian CSC signature genes.

All these and several other data indicate that the gene expression profile and the presence of specific mutation/genomic aberration may help to identify the culprit of the cancer stem cell compartment. The improvements in our understanding of the molecular and stem cell basis of ovarian cancer should lead to more efficacious treatment.

3.) Minor comments: 1…and critical discussion…" should be "..and a critical discussion…" 2.…"fetal disease" is this correct or a spelling error?

Answer: Spelling errors were corrected as suggested (1. a critical discussion; 2. fatal disease)