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Ovarian cancer stem cells

*Minireview***

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Because of its *semi-solid* character in dissemination and growth, advanced ovarian cancer with its hundreds of peritoneal tumor nodules and plaques appears to be an excellent *in vivo* model for studying the cancer stem cell hypothesis. The most important obstacle, however, is to adequately define and isolate these tumor-initiating cells endowed with the properties of anoikis-resistance and unlimited self-renewal. Until now, no universal single marker or marker constellation has been found to faithfully isolate (ovarian) cancer stem cells.

As these multipotent cells are known to possess highly elaborated efflux systems for cytotoxic agents, these pump systems have been exploited to outline putative stem cells as a side-population (SP) via dye exclusion analysis. Furthermore, the cells in question have been isolated via flow cytometry on the basis of cell surface markers thought to be characteristic for stem cells.

In the Vienna variant of the ovarian cancer cell line A2780 a proof-of-principle model with both a stable SP and a stable ALDH1A1$^+$ cell population was established. Double staining clearly revealed that both cell fractions were not identical. Of note, A2780V cells were negative for expression of surface markers CD44 and CD117 (c-kit). When cultured on monolayers of healthy human mesothelial cells, green-fluorescence-protein (GFP)-transfected SP of A2780V exhibited spheroid-formation, whereas non-side-population (NSP) developed a spare monolayer growing over the healthy mesothelium. Furthermore, A2780V SP was found to be partially resistant to platinum. However, this resistance could not be explained by over-expression of the "excision repair cross-complementation group 1" (*ERCC1*) gene, which is essentially involved in the repair of platinated DNA damage. ERCC1 was, nonetheless, over-expressed in A2780V cells grown as spheres under stem cell-selective conditions as compared to adherent monolayers cultured under differentiating conditions. The same was true for the primary ovarian cancer cells B-57.

In summary our investigations indicate that even in multi-passaged cancer cell lines hierarchic government of growth and differentiation is conserved and that the key cancer stem cell population may be composed of small overlapping cell fractions defined by various arbitrary markers.

Key words: ovarian cancer, stem cell, side-population, mesothelium-co-culture, flow cytometry, ERCC1

A rapidly growing body of evidence is lending support to the idea that human cancer can be regarded as a stem cell disease. According to the "cancer stem cell hypothesis", tumor growth is not viewed as a simple monoclonal expansion of transformed cells, but as being decisively driven by a minority of cellular components that display stem cell-like properties. These cells maintain their inherent capacity for unlimited self-renewal through asymmetric division and have an ac-

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quired malignant phenotype characterized by uncontrolled proliferation, the ability to form metastases and to generate differentiated, although aberrant, progeny. The multipotent character of these cells may explain the histological heterogeneity often found in tumors [1–4]. This working model is supported by the following experimental observations: (i) only a small fraction of the entire tumor cell population is endowed with tumorigenic potential when transplanted into immunodeficient mice, (ii) tumors emanating from this tumorigenic cell fraction mirror the full phenotypic heterogeneity of the parent tumor and contain mixed populations of both tumorigenic and non-tumorigenic cancer cells, (iii) tumorigenic cancer cells are characterized by a distinctive profile of surface markers and can be reproducibly isolated. Although a number of different clue markers have been described, the profile of surface markers appears to differ among the various entities of solid tumors and probably from patient to patient. In ovarian cancer CD44, CD24, CD133, CD117 (c-kit) and aldehyde dehydrogenase isoform 1 (ALDH1A1) have been proposed to contribute to the stem cell surface signature and could be used to recover these cells from the entire bulk of cancer cells [5-9]. Especially in ovarian cancer it should be emphasized that the expected membrane surface signature may vary considerably according to the various histological subtypes and possibly between type 1 (frequent K-Ras/BRAF mutations) and type 2 (frequent p53 mutations) molecular biological backgrounds. Furthermore, it should be noted that functional assays performed to verify stemness in various cancer cell populations isolated by virtue of the aforementioned markers yielded controversial results [10].

Considering that drug resistance is a further essential attribute of cancer stem cells, separation based on expression of various membrane-spanning ATP-binding cassette transporters, such as the multidrug-resistant gene 1 (MDR1 also known as ABCB1 and P-glycoprotein) and breast cancer-resistance protein 1 (BRCP1/ABCG2), is regarded as a valuable tool for recovery of these cells. Within bone marrow, researchers have defined a subset of verapamil-sensitive ABCG2-expressing cells exhibiting the ability to efflux the lipophilic dye Hoechst 33342. This subset has been described as a side-population (SP), which indeed has been shown to exhibit stem cell-like features [7, 11-13]. In a large number of solid tumors and established cancer cell lines SPs have been evidenced either by Hoechst 33342 or Dye Cycle Violet (DCV), another cell-permeable DNA-binding dye evacuated by the same transporters [14]. This article reports on our experience with demonstration of the surface markers mentioned above and dye exclusion assays to delineate tumorigenic cells out of various established ovarian cancer cell lines and primary cultured ovarian cancer cells. During these investigations the Vienna-variant of the cell line A2780 (A2780V) emerged as the most valuable model for studying the various subpopulations of cells in the context of a hierarchic concept of cancer growth and dissemination.

Why ovarian cancer could be an excellent *in vivo* model for studying tumor stemness? Ovarian cancer can be regarded as a *semi-solid* malignant disease that disseminates via the peritoneal fluid in the form of single cells, clusters of some cells or even small spheroids throughout the whole peritoneal cavity. Several cell surface molecules such as CD44, mesothelin and others have been proposed to be decisively involved in the adhesion and implantation of ovarian cancer cells on the single-layered peritoneal mesothelium [15,16]. More than 75% of ovarian cancers are diagnosed at an advanced stage exhibiting a considerable carcinosis of the visceral and parietal peritoneum [17]. As depicted in Figure 1, hundreds of tumor nodules and confluent plaques of different sizes can be found side by side during primary debulking surgery. While the very small nodules of 1 to 2 mm in diameter behave like spheroids, that are in loose contact with the underlying mesothelium and can be easily drawn away without causing bleeding, the more prominent nodules or plaques are tightly adherent to the peritoneum and have their own blood supply.

Considering this mode of dissemination together with the assumption that each of these hundreds of cancer nodules originates from at least one "tumor-initiating" cancer cell exhibiting stem cell(-like) properties, we hypothesize that advanced ovarian cancer can serve as a valuable human *in vivo* model for the study of tumor stemness. As *in vitro* investigations have elucidated that the number of cancer stem cells is enriched in anchorage-independent growing spheres and subsequent spheroid formation is associated with cell differentiation [18], it should be assumed that the highest percentage of cancer stem cells can be demonstrated in the very small tumor nodules obtained from ovarian cancer patients. To corroborate these *in vitro* findings, we are currently collecting series of tumor nodules of different sizes in a number of patients. These tumors will be investigated with immunohistochemistry and RT-PCR for stem cell-related membrane surface markers, such as CD133, CD44, CD24, CD117 (c-kit) and ALDH1A1 as well as for the classical stem cell self-renewal signature (e.g. Nanog, Oct-4, Sox-2) and other putative determinants of pluripotency such as Stella, FGF4, BMP4 and Rex-1. Furthermore, primary ovarian tumors and their respective peritoneal metastases will be "disassembled" by enzymatic digestion. From the single-cell suspension of isolated cancer cells the side- (SP) and non-side-population (NSP) obtained by dye exclusion assay as well as the various fractions determined by means of the above cited cell surface markers will be isolated by FACS and used for *in vitro* and *in vivo* functional- and repopulation assays to determine the clonogenicity or tumorigenicity of the distinct fractions and to elucidate their putative stem cell-(-like) character.

The clinical course of ovarian cancer in patients also suggests that stem cell-driven repopulation is a prominent phenomenon in this disease. Although, even in advanced disease, the rate of complete clinical remission is very high following primary debulking surgery and first line platinum-based chemotherapy,

the recurrence rate is as high as more than 80% in these patients. One intriguing trait of ovarian cancer is that relapses occurring later than six months after termination of the last platinum-based chemotherapy cycle can be successfully re-challenged with platinum drugs. As a rule, however, the intervals between recurrences become progressively shorter, until platinum sensitivity is lost (Figure 2). It is conceivable that during various treatment courses, tumor stem cells known to exhibit constitutive resistance to cytotoxic agents increase in their relative number as a result of a continuous selection towards clinical chemoresistance. On the other hand, the increase in the stem cell population during tumor progression could also be due to an accumulation of genomic mutations in these cancer stem cells, frequently limiting their ability to differentiate into more chemosensitive progenies [reviewed in ref. 19].

An additional, but indirect, hint that ovarian cancer is a disease that is especially related to cancer stemness comes from the results of recent clinical trials, investigating the effectiveness of anti-angiogenic therapy via VEGF blockade with bevacizumab, a humanized monoclonal antibody. Two large double-blinded, randomized clinical trials proved to be effective when this monoclonal antibody was given as maintenance therapy after primary chemotherapy for 15 and 12 months, respectively. When considering the Kaplan-Meier progression-free survival curves for both studies, the survival benefit of the patients in the experimental arm was seen to be highest at the end of treatment followed by an approximation of the survival curves for the control- and experimental arms two to three months after discontinuation of the bevacizumab treatment [20,21]. These results are exemplary for ovarian cancer and have not been reported with such consistency for other tumor entities. All this makes it tempting to speculate that tumor initiation can be suppressed as long as anti-VEGF treatment is continued. On the one hand, bevacizumab activity can be explained as simple inhibition of micro-vessel formation with consecutive suppression of cancer growth caused by reduced blood supply. On the other hand, there is a growing body of evidence to show that VEGF plays a key role in the cross-talk between the so-called vascular niche and tumor-(initiating) stem cells. The paramount significance of adequate interaction between the vascular niche and stem- and precursor cells in determining the fate of these cells was demonstrated long ago in the hematopoietic system. Such indirect cancer stem cell targeting via the vascular niche by means of VEGF antagonism is considered an attractive treatment option, all the more so because until now there is no consensus signature of cell surface markers that unequivocally allows recognition and thus direct targeting of (ovarian) cancer stem cells [22].

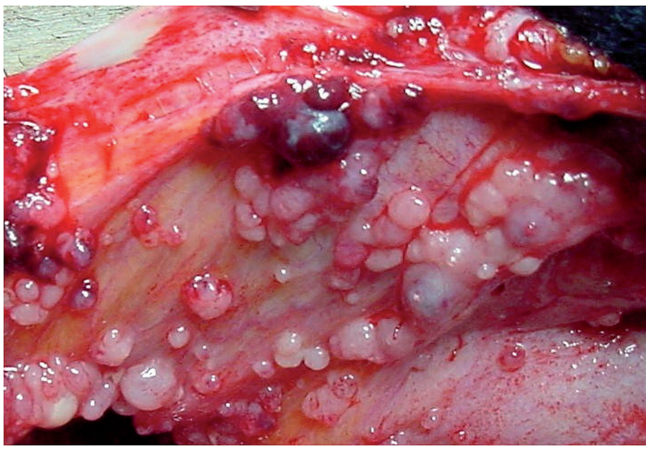


Figure 1. Typical peritoneal dissemination in advanced ovarian cancer.

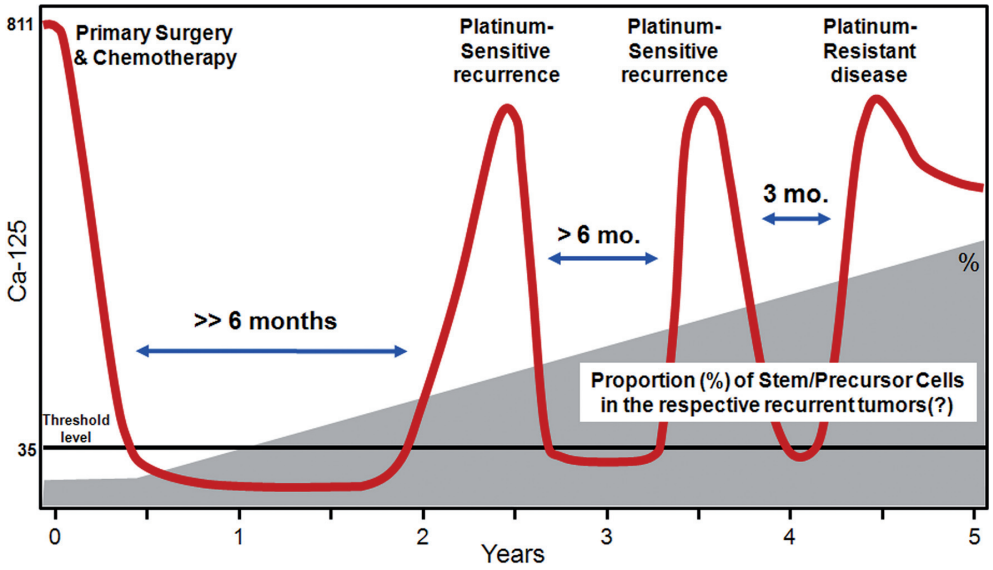


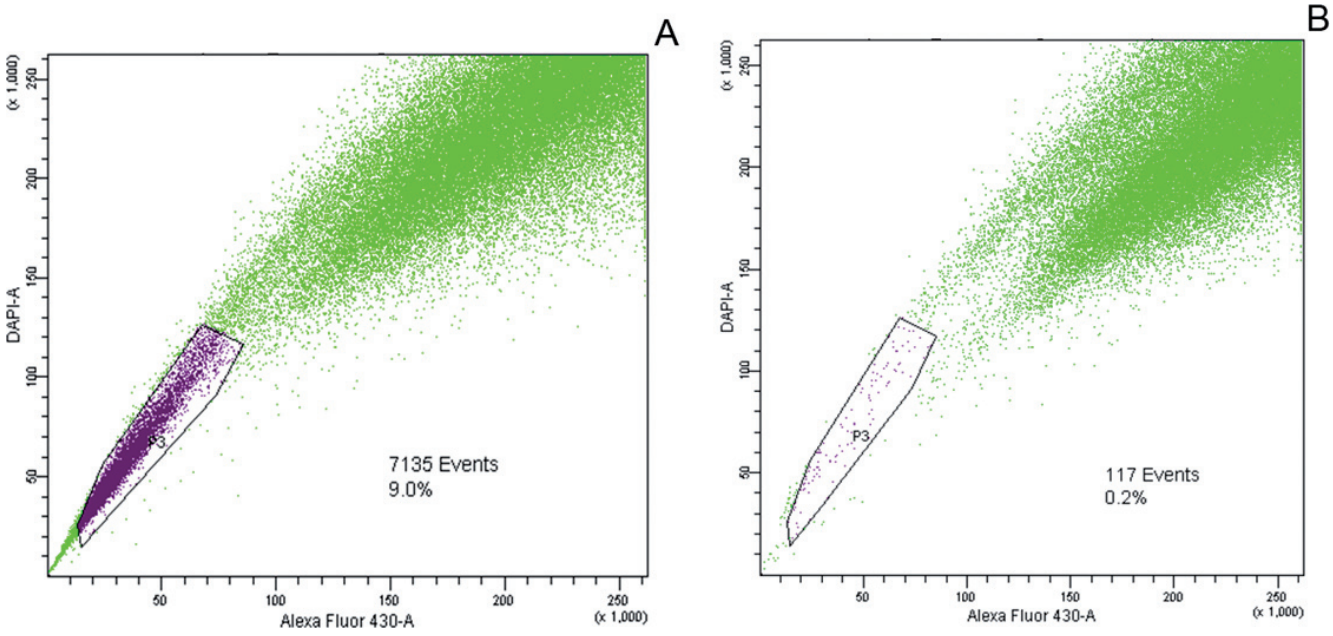
Figure 2. Classical clinical course with a number of recurrences and the hypothetic continuous increase in the relative number of stem cells.

Dye exclusion assays to separate side- from non-side populations. SPs have been successfully identified in several established cell lines derived from a variety of different tumor entities [23-25]. This implies that the hierarchical proliferative pattern according to the "cancer stem cell theory" is obviously conserved in multi-passaged human cancer cell lines. This prompted us to investigate all breast and ovarian cancer cell lines available in our institutions for the presence of SPs using the Hoechst 33342 and the DCV exclusion methods. The L-type calcium channel blocker verapamil was used to inhibit ABC drug transport in control experiments to accurately discriminate the SP cell fraction.

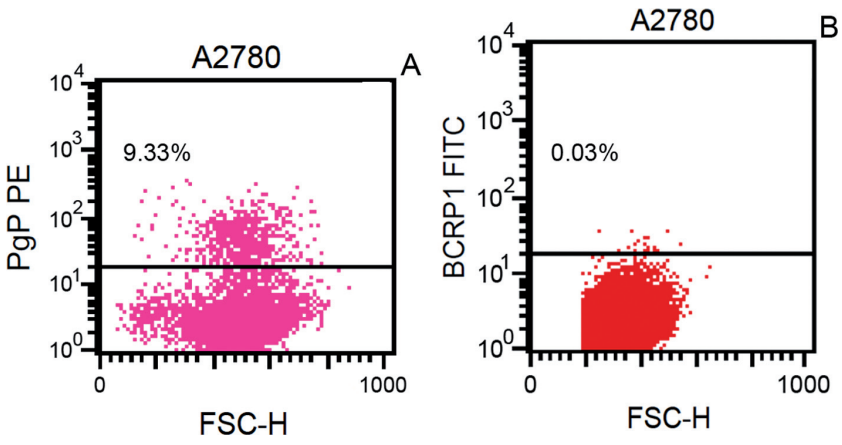
We were able to identify SPs in the ovarian cancer cell lines A2780, A-6000, OVCAR3, B2/92 and the breast cancer cell lines BT20, SKBR3, MCF-7 and T24D. In contrast, no SPs were discriminated in HOC7 and HTB77, even after numerous attempts. However, in most of the cell lines (i.e. OVCAR3, B2/92, SKBR3, MCF-7, and T24D), the SP demonstration was inconsistent, whether with Hoechst 33342 or DCV exclusion. Thus, in these cell lines SP phenotype was unsteady and the percentage of the fraction of cells designated as SP varied considerably from assay to assay in the same cell line. This may be due to even slight differences in culture conditions, the procedure used to expand the cells and/or the number of uninterrupted series of passages in the actual experimental setting. Ince et al. recently evidenced that the propagation potential, i.e. the tumorigenic and metastatic phenotype of tumor cells, is highly dependent on the culture medium and the nature of the artificial culture surface [26,27]. These findings furthermore corroborate the concept that *in vivo* the tissue- or vascular niche is one of the most decisive factors in determining the fate of cancer stem cells.

The most consistent results regarding the separation of SPs from NSPs were yielded in the ovarian cancer cell line A2780, especially the Vienna variant of this cell line (A2780V) (Figure 3A + B). When we analysed this cell line for the various ABC transporters, we found that a fraction of (9.30% ± 1.91% of) the A2780V cells abundantly expressed P-glycoprotein but not ABCG2 (Fig. 4A + B). While in the original A2780 cell line purchased from Sigma-Aldrich (#93112519) a consistent, smaller SP of cells was demonstrated, this SP proved to be insensitive to verapamil clearance. This phenomenon of verapamil insensitivity despite a clearly delimitable SP has also been found in a number of other cell lines and may be due to the expression of ABC transporter variants exhibiting impaired sensitivity to verapamil. In these cases it would be advisable to try other blocking-agents like the broad efflux inhibitor (e.g. reserpine and GF120918) or fumitremorgin C, which, in contrast, is endowed with the ability to selectively inhibit the ABCG2 transporter. Investigations into the functional characteristics of these putative SPs exhibiting resistance to verapamil and other efflux inhibitors are currently in progress.

An adapted *in vitro* co-culture model simulating ovarian cancer implantation. As mentioned earlier, the classical dissemination of ovarian cancer remains confined to the abdominal cavity, where the healthy peritoneum is seized by hundreds of tumor implants in the form of nodules of different size and confluent tumor plaques of some centimeters in diameter. To study adhesion and implantation of tumor cells on the normal peritoneum and especially to investigate whether



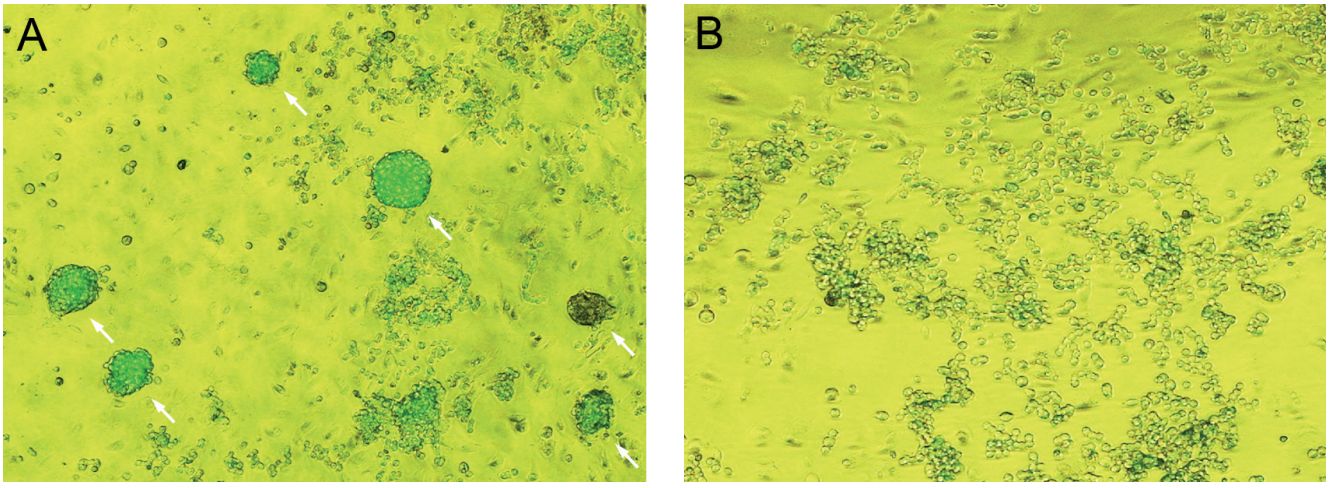
Figures 3 (A): Side-population (SP) in blue, non-side-population (NSP) in green. (B): SP fraction is displaced to the bulk of NSP through efflux pump blocking with verapamil *(SP clearance).*



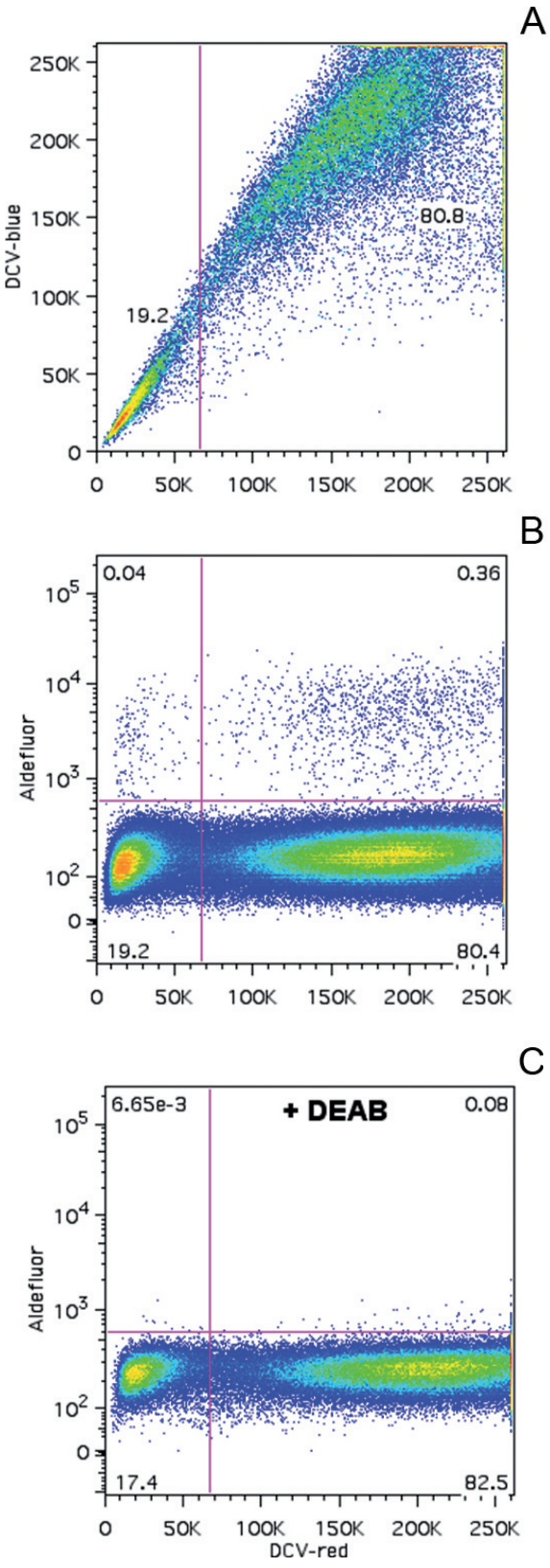
Figures 4 (A): FACS analysis of expression of the membrane efflux pump p-glycoprotein (MDR1) in the A2780V cell line *(violet cell population = 9.30%)*. **(B): FACS analysis of expression of the membraneous BCRP1 (ABCG2) in the A2780V cell line** *(red cell population = practically absent)*.

or not there are differences in these processes between SPs and NSPs, we developed an *in vitro* co-culture model, which was essentially based on two main characteristics: firstly, the primary culture of healthy mesothelial cells as a monolayer with their classical uniform cobblestone appearance after confluence and, secondly, the stable transfection of the tumor cells with green-fluorescence-protein (GFP) to discriminate between healthy mesothelium and cancer cells during co-culture. Healthy mesothelial cells were obtained from omental tissue from patients undergoing elective abdominal surgery. Isolation of these cells and culture conditions have been described elsewhere [28]. The mesothelial origin of the cultured cells was verified by the presence of desmosomes and surface microvilli as well as by their coexpression of vimentin and the cytokeratin types 8 and 18 [29]. For stable GFP transfection A2780V cells were cultivated under growth-factor reduced conditions (1% serum) and incubated with a lentivirus (SMARTvector, Thermo Scientific), containing the open reading frame of turbo-GFP (Evrogen, Moscow, Russia). Optimal transfection was achieved with a multiplicity of infection of five viruses/cell and the use of 6 µg/mL polybren to enhance efficiency of virus transmission. After overnight incubation, medium was changed and 48 hrs post-transfection cells were selected for seven days using 3 µg/mL puromycin.

In GFP-transfected A2780V cells an SP-fraction of the same size as the non-transfected A2780V was detected. As shown in Figures 5 A and B, SP cells co-cultured on confluent mesothelial monolayers developed small clusters and progressed rapidly to small spheroids. In contrast, cells derived from NSPs unusually showed rudiments of spheroid-like growth and generally grew as a fragmentary monolayer of GFP-positive cancer cells over the mesothelial cobblestone. These findings



Figures 5 (A): Co-culture of GFP-tagged SP cells on healthy human mesothelial cell monolayer showing development of spheroids. (B): GFP-tagged NSP cells growing as a fragmentary monolayer over the mesothelial cobblestone.



Figures 6 (A +B): Distribution of ALDH1$^+$ subpopulation between SP and NSP. (C) Negative control for ALDH1 obtained by DEAB blocking.

clearly indicate that SPs, at least from the cell line A2780V, mirror the *in vivo* ovarian cancer growth far more faithfully than do the bulk of the NSP cells.

Are side-populations identical to other putative cancer stem cell populations? Our next goal was to establish the relationship between the SP from A2780V cells and the other subpopulations of that cell line characterized by virtue of cell surface markers. The standard variant of CD44, which was highlighted by Gil Mor and co-workers as one of the most prominent candidate surface antigens for defining stemness in ovarian cancer [30, 31] and which was earlier described as being crucially involved in the adhesion of free-floating cancer cells or cell clusters to the peritoneum [15], could not be detected in the A2780V cell line. The same was true for the surface marker CD117 (c-kit). The expression of this tyrosine kinase oncoprotein has recently been implicated in ovarian cancer stemness in combination with CD44 positivity. As few as one hundred CD117$^+$/CD44$^+$ cells were able to serially propagate their original tumor, whereas 10^5 CD117$^-$/CD44$^-$cells remained non-tumorigenic [5]. In the A2780V cell line a small fraction of CD133$^+$ cells was irregularly demonstrated. This cell surface molecule was recently reported to be determinative for ovarian cancer-initiating (stem) cells, either alone or in association with the ALDH1A1$^+$ phenotype. The experimental setting of this report [32] was based on the gradual loss of cells tagged by stem cell markers after serial *in vitro* passages and the attempt to rescue these cells in sphere cultures under serum-free and anchorage-independent conditions. These investigations revealed that cells with the CD133$^+$/ALDH1A1$^+$ marker combination were far more likely to initiate sphere formation than were cells with other surface signatures. The predominant role of the ALDH1A1$^+$ CD133$^+$ cell population in ovarian cancer stemness is further corroborated by the work of Silva et al., who showed that ALDH1A1$^+$/CD133$^+$ cells exhibited increased angiogenic capacity as compared with the bulk of tumor cells and that the presence of these cells in primary tumor specimens correlated with reduced disease-free and overall survival in ovarian cancer patients [33]. In line with this, ALDH1A1 positivity for itself has also been found to characterize a subpopulation of cells with cancer stem cell(-like) properties in ovarian cancer [34].

Interestingly, in A2780V we were able to demonstrate a small ALDH1A1$^+$ population that was, however, nearly equally distributed in both the SP and the NSP, indicating that the SP and the ALDH1A1 cell fraction only partially overlap and are ultimately distinct populations (Figure 6 A-C). We are currently investigating whether there is a significant functional difference between the ALDH1A1$^+$-SP and the ALDH1A1$^+$-NSP cells.

Unless verified in other experimental systems, our findings should be considered proof of principle with exclusive value for the cell line A2780V, nevertheless. It is to be expected that expression of these stem cell markers would be highly variable between the various established cell lines and especially between fresh ovarian cancer cells from different donors.

Platinum resistance may be related to a small fraction of cancer cells. Platinum-containing anti-cancer drugs show

by far the highest effectiveness in the treatment of patients with ovarian carcinomas. Thus, it was obvious that we should test platinum sensitivity in A2780V SP and their NSP counterpart. Our findings indicate that the SP is less sensitive to cisplatin (LD 50: 11.5 μg/ml) than is the NSP (LD 50: 7 μg/ml). Platinum-resistance, however, is multifactorial and numerous molecular pathways are known to be involved in that intrinsic or acquired drug resistance. Among these pathways, those conferring an increased capacity for DNA repair, enhanced drug efflux and/or drug inactivation are thought to play the most - prominent role [35].

In DNA repair, the "excision repair cross-complementation group 1" (ERCC1) protein has the important task of incising the DNA strand at the 5' site relative to platinated DNA damage. In line with this, overexpression of ERCC1 has been associated with clinical resistance to cisplatin in several tumor entities including ovarian cancer [36,37]. This prompted us to investigate the expression of the *ERCC1* gene by RT-PCR in A2780V SP and NSP as well as in ALDH1A1$^+$ and ALDH1A1$^-$ A2780V subpopulations. However, neither in the SPs nor in the ALDH1A1$^+$ cell fractions were we able to reveal a significant difference in *ERCC1* mRNA expression as compared with their respective counterparts, namely the NSPs and the ALDH1A1$^-$ subpopulations. Hence, we conclude that the demonstrated relative resistance to cisplatin in A2780V SPs is not caused by an over-expression of ERCC1. We are currently investigating the role of an increased efflux of platinum drugs out of SP cells as compared to NSP cells to explain the cisplatin resistance in A2780V SPs. It is worth noting that in the A2780V cell line a very small subfraction of CD133$^+$ cells has been inconsistently identified. Although mRNA recovery from the small number of isolated cells was very low, preliminary data indicate that CD133$^+$ cells exhibit a roughly three-fold *ERCC1* expression as compared with CD133$^-$ cells.

Furthermore, in A2780V cells and in primary cultured ovarian cancer cells termed B-57, the expression of the *ERCC1* gene was assessed in non-adherent spheres cultured under stem cell-selective conditions and in adherent monolayers cultured under conventional differentiating conditions. In contrast to A2780V, B-57 cells lacked a distinct SP and also an ALDH1A1$^+$ subpopulation. Interestingly, in both ovarian cancer cell lines, we found that a markedly increased expression of *ERCC1* mRNA is prevalent in cancer spheres as compared with the cells grown under adherent conditions. In A2780V and B-57 cells a 3.75-fold and 4.15-fold higher expression of *ERCC1*, respectively, was revealed in spheres relative to the monolayer cultures. These findings tempt us to speculate that either those cells are capable of initiating sphere-growth and their respective clonal progenies overexpress ERCC1 or that *in vitro* culture conditions decisively regulate expression or silencing of the *ERCC1* gene. Nonetheless, all this underlines that several mechanisms leading to platinum resistance may coexist in a same cell line (e.g. A2780V) and, furthermore, that these various mechanisms can be covered by multiple distinct subpopulations in one established cancer cell line or conceivably in one individual primary ovarian cancer [38].

Conclusion and future prospects. Even in multi-passaged cancer cell lines hierarchic government of growth appears to be conserved. However, our results also indicate that it remains unclear whether this hierarchy is covered in a simple *top-down* manner by one single superordinated cell population or is warranted by different subclones of cells. Essentially, this issue concerning multiple subpopulation endowed with stemness, is highly dependent on how stem cells are finally defined with regard to the chosen marker constellation or the used isolation technique. It is even more than likely that there is considerable variability in the signature of stem cell markers, not only between the various tumor entities but also between the individual cancers. Furthermore, it seems that the properties inherent to stemness, such as resistance to cytotoxic agents, are not always caused by one distinct molecular mechanism, but may be differently generated in various cell populations isolated from one single cell line and probably from one particular tumor. In addition, depiction of a distinct SP in the dye exclusion assay is not exclusively dependent on the presence of the membranous drug efflux transporter ABCG2, but can also be identified through dye efflux via P-glycoprotein. Moreover, expression of these pump systems does not appear to be stable, but is supposed to be highly dependent on the culture conditions in cell lines and on the close micro-environment, the so-called stem cell "niche" in primary tumors.

Our future understanding of oncologic principles and our endeavors to treat cancer must take into consideration that a primary tumor as well as all its individual metastases behave like an organ and its injuries - mostly due to therapeutic efforts – may cause recruitment of stem cells similar to that occurring during wound healing and *regeneration*. It remains completely open, whether extension of the stem cell pool is due to so-called "emergency symmetric division" of stem cells [39] or to a dedifferentiation of precursor or partially differentiated cells. Stemness and especially recruitment of stem cells have been closely linked to the dynamics of epithelial-mesenchymal transition (EMT) and mesenchymal-epithelial transition (MET) (see accompanying paper of Hatina, this issue of Neoplasma). Both these processes, which cause a tremendous change in cell fate, are phenomena that are not dependent on genomic mutations but on a reversible epigenetic regulation, including governance via microRNAs. In summary, tumor stemness together with its related molecular programs for EMT and MET keeps cancer cells in a state of multidirectional instability and allows rapid adaptation to changing environmental conditions to the benefit of tumor survival and progression. This *"plasticity of cancer"* may be one major reason why until today malignant tumors continue to successfully escape anti-tumor treatment.

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