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Characteristics of *in vitro* model systems for ovarian cancer studies

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ABSTRACT

Nowadays, targeted therapy plays a growing role in oncological treatment. In ovarian cancer, particularly promising results are achieved with poly (ADP-ribose) polymerase (PARP) inhibitors. Recent clinical trials have shown that PARP inhibitors can result in significantly longer progression-free survival. These results encourage the search for other targeted therapies and bring hope that ovarian cancer can soon become a manageable chronic disease. The main problem in ovarian cancer research is the heterogeneity of this disease. Recent studies have shown that different histological types of ovarian cancer can originate from distinct tissues. According to the recent knowledge, "ovarian cancer" is an artificial term for distinct invasive malignancies localised within the pelvis. Genetic and immunophenotype analyses have shown that high-grade serous ovarian cancer, the most frequent histological type and the one with the worst prognosis, originates mainly from fallopian tube epithelium, while endometrioid and clear-cell cancers originate from the endometrium. For these reasons, in basic and preclinical studies on ovarian cancer, one has to carefully choose a well-defined model system, corresponding to the histological type of interest. In this article, we discuss ovarian cancer cell lines most frequently used in *in vitro* studies. Our aim is to indicate the advantages and disadvantages of different models, encompassing primary and established cell cultures, two- and three-dimensional models, etc. In particular, we would like to alert researchers to the fact that the most popular cell lines SKOV3 and A2780 do not represent a suitable model for studies on high-grade serous ovarian cancer.
Key words: ovarian cancer, *in vitro* models, ovarian cancer cell lines, 3D cell culture, high-grade serous ovarian cancer, chemoresistance

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Introduction

Ovarian cancer is characterised by high mortality. The reasons for this include late diagnosis, asymptomatic early stages of the disease, and the lack of effective tools for early diagnosis and screening.

The standard treatment of advanced ovarian cancer includes surgery as well as paclitaxel- and platinum-based chemotherapy. Most patients respond very well to the treatment, but relapse of the disease and increasing chemoresistance are problems that frequently occur. Usually, there are several relapses in the course of ovarian cancer, interwoven with disease-free periods. Patients with relapse are mainly treated with chemotherapy until the resistance develops. Recently, agents directed at specific biological targets are being introduced into the treatment of recurrent ovarian cancer. Clinical studies indicate that the survival time of patients with ovarian cancer can be significantly prolonged by drugs such as poly ADP-ribose polymerase (PARP) inhibitors (PARPi) or, to a lesser extent, the anti-angiogenic agent bevacizumab. Therefore, ovarian cancer has a chance to become a chronic disease that can be successfully controlled for years [1]. Targeted drugs, however, are currently very expensive and are therefore recommended for a limited number of indications.

In many countries, they are not reimbursed and therefore are not yet widely used in clinical practice [2].

Positive results of clinical trials with new biological drugs encourage further exploration. A significant problem in ovarian cancer research is the heterogeneity of this disease [1]. To properly design an experiment and obtain reliable results, it is crucial to specify what histological type of ovarian cancer is to be studied and to select the relevant model.

Heterogeneity of ovarian cancer

Several histological types of ovarian cancer have been distinguished; the most common are serous, endometrioid, clear-cell, and mucinous cancers. Classical theory assumed that all these tumours originate from a single layer of mesothelial epithelium covering the ovary (ovarian surface epithelium, OSE). It was assumed that the initiation of the neoplastic process occurs in OSE under the influence of cyclical stimulation by hormones, cytokines, and growth factors, secreted in the process of ovulation and tissue healing, after the oocyte release. Differentiation of the tumour in the direction of particular histological type was to be a secondary process.

In 1999, Dubeau challenged the above scenario and proposed that most cases of ovarian cancer originate from epithelia lining structures originating from Müller's ducts, i.e. the cervix, uterus, and fallopian tubes [3]. Over time, experimental evidence was gathered to support the Dubeau theory. Substantial immunophenotypical, genetic, and molecular differences between individual histological types of ovarian cancer have been demonstrated. For example, the majority of serous cancers have common features with the Müllerian epithelium lining the fallopian tubes, e.g. expression of HOXA and PAX8 proteins. Expression of these proteins is not observed in OSE. It is currently assumed that the majority of high-grade serous ovarian cancer (HGSOC) cases originate from malignant epithelial cells of the fallopian tube, which are re-implanted on the ovary and/or peritoneum surface. This is explained by the rapid spread of HGSOC. In turn, low-grade serous ovarian cancers (LGSOC) are derived from ovarian inclusion cysts and are of diverse origin. Some of these cysts arise due to OSE invagination, and some as a result of implantation of the fallopian tube epithelium. Under the influence of the local microenvironment, the cancer process may initiate in the cyst. Endometriotic foci, e.g. the fragments of the endometrium that have migrated up the fallopian tube and nested on the surface of the ovary, are considered as precursors of endometrioid and clear-cell cancers. This is supported by, among others, the protective effect of tubal ligation, which reduces the risk of development of these histological types, due to blocking of the migration path of their precursors. Mucinous tumours have many morphologic features in common with gastrointestinal cancers and glandular cervical cells. Their origin is still unexplained [4, 5].

Individual histological types of ovarian cancer also differ in molecular profile. HGSOC is characterised by a high percentage of *TP53* gene mutations (over 95% of cases) and a loss of *BRCA1* or *BRCA2* gene function. There are no mutations of other genes in HGSOC, while the high variability of DNA copy number in the entire genome is characteristic (copy number variation, CNV). LGSOC are characterised by the presence of *BRAF* or *KRAS* gene mutations. Endometrioid and clear-cell cancers show instability of microsatellite sequences and *PIK3CA* and *PTEN* gene mutations. In clear-cell carcinoma, *ARID1A* gene mutations additionally occur, and in the endometrioid cancer — *CTNNB1* gene mutations. Mutations of the *KRAS* gene are characteristic for the mucinous type [5, 6].

In 2004, Shih and Kurman proposed a new classification, dividing ovarian cancers into two types (Table 1). Type II includes HGSOC; sometimes poorly differentiated, clear-cell carcinomas are also included. Type II cancers are usually diagnosed in stage III or IV according to FIGO and have a very poor prognosis. They account for about 75% of all cases. Type I consist of the remaining histological types. Their diagnosis is made in earlier stages, and the prognosis is favourable [7].

In conclusion, the knowledge accumulated over the last dozen or so years has redefined the understanding of the disease traditionally known as ovarian cancer. Many data indicate that it is an artificial term that includes various pelvic neoplasms that have separate histogenesis, other mutational trajectories, and a diverse clinical picture. The knowledge about the heterogeneity of ovarian cancers should therefore be taken into account in both clinical practice and research design.

In vitro models used in ovarian cancer research

The main model in basic and preclinical studies on ovarian cancer are cell lines maintained as *in vitro* culture. Cell lines can be stabilised (capable of an infinite number of *in vitro* divisions) or primary, i.e. freshly taken from the body. The most common objects of research are cancer cells, normal precursor cells of a given cancer, and stem cells.

The limitation of the cellular culture model is the loss of tissue histology, lack of endocrine, paracrine, and nerve signalling, and lack of gradients of nutrients and other substances found in the living organism. However, the enormous advantages, including ease to propagate

Table 1. Classification of ovarian cancers based on clinical and molecular features [7]

Type I (25% of cases)	Type II (75% of cases)
Low-grade serous ovarian cancer (LGSOC) Clear-cell cancer Endometrioid cancer Mixed cancer Mucinous cancer	High-grade serous ovarian cancer (HGSOC)
Derived from precursor lesions (borderline tumours, endometriosis)	Derived from serous tubal intraepithelial cancer (STIC)
Somatic mutations in *KRAS*, *BRAF*, *PTEN*, *PIK3CA*, *CCTNB1*, *ARID1A*, *PPP2R1A* genes	95% of cases have *TP53* gene mutation 40–50% of cases have inactivated *BRCA* genes
Slow growth	Fast growth and high aggressiveness
Limited to the ovary	Rarely limited to the ovary
Diagnosed mostly at I and II clinical stage according to FIGO	Diagnosed mostly at III and IV clinical stage according to FIGO
Low sensitivity to chemotherapy	High sensitivity to chemotherapy
Rare relapses	Frequent relapses
Favourable prognosis (80% of 5-year survival)	Unfavourable prognosis (10% of 5-year survival)

cells, stability, and reproducibility of the model, determine its popularity and irreplaceable role.

Primary cell lines

Short-term cultures of freshly *ex-vivo* harvested cells are a valuable model, especially when correlation of the results of *in vitro* studies with clinical data is possible. However, they have many limitations, such as the need for every-time preparation of cells from biological material, slow *in vitro* growth, and limited viability. The reproducibility of this model is low due to the fact that the cells come from a different donor and from another cancer each time. In addition, the clonal selection of cells progresses during the culture, and their initial composition changes.

In cancer research, the primary cultures of epithelium considered to be the precursor tissue of a given cancer are often used as a control. In ovarian cancer, ovarian epithelial cells — OSE — have been used for this purpose for a long time. Because the theory that some HGSOC originate from the fallopian tubal epithelium has been recognised, it seems reasonable to use these epithelial cells as a control. However, studies that only use OSE are still being published. An even greater mistake is the use of whole ovarian fragments containing stromal elements and germinal cells. According to current knowledge, the original tissues for various ovarian cancers are: fallopian tubal epithelium, ovarian epithelium, endometrium, and endometriosis, and possibly the intestinal epithelium or epithelium covering the peritoneum. Examples of primary and immortalised control epithelia are presented in Table 2.

The normal cells can be maintained in *in vitro* culture for 6–8 weeks. Over time, the loss of specific markers, followed by apoptosis or the aging process (senescence), is observed. Senescent cells are huge, have numerous vacuoles, and stop dividing.

Primary cultures of ovarian cancer cells can be derived from solid tumours or peritoneal fluid. In the first case, the preparation starts with the mechanical dissociation of the tissue and the enzymatic digestion of extracellular matrix proteins. In case of peritoneal fluid, the initial elimination of erythrocytes is indicated, e.g. by density gradient centrifugation. From primary culture, fibroblasts can be eliminated through so-called differential trypsinisation — fibroblasts are detached from the surface of culture vessel after about two minutes of reaction with trypsin, and tumour cells show stronger adhesion. Primary cultures of ovarian cancer cells are relatively easy to derive compared to other cancers — their advantage is high viability, strong adhesion to the surface, and rapid cell division. In case of obtaining material from patients after chemotherapy, cell viability may be limited and their *in vitro* growth slowed down. It should also be remembered that these cells do not represent all cell clones present in the tumour before the start of therapy due to selection processes [18].

Primary cultures of tumour cells often die after about 2–3 months of *in vitro* maintenance. In some cases, stable cell lines can be established. In our practice, with the material from the peritoneal fluid from eight patients, we managed to derive one stable tumour line [19]. Recently, Ince et al. developed a special culture (growth) medium (Ovarian Carcinoma Modified Ince, OCMI) for the derivation of stable lines of ovarian cancer cells. This medium is based on commercially

Table 2. Examples of primary and immortalised control epithelium considered as ovarian cancer precursor tissue

Line name	Source	Type	Modification	Evaluated markers	References
FT33-shp53-R24C	FT	I	Retroviral transfer of hTERT, p53, CDK4R24C or shRNA	CK-7, PAX8	Creative Bioarray [8]
HFTEC	FT	P	–	CK-8/18, CK-14, CK-19	Life Line Cell Technology [9]
HOSEpiC	OSE	P	–	CK-14, CK-18, CK-19	ScienCell Research Laboratories [10]
Human Primary Ovarian Surface Epithelial Cells	OSE	P	–	CD326, E-cadherin	ABM [11]
HOSE1, HOSE2	OSE	I	Lentiviral transfer of hTERT, Cdk4, or cyclin D1	–	Sasaki et al., 2009 [12]
iFTSEC283	FT	I	–	–	Gjyshi et al., 2018 [13]
IOSE-29, IOSE-80	OSE	I	Simian virus 40	α-, β-, γ-catenin, CA125, E-cadherin, F-actina, pan-cytokeratin	Auersperg et al., 1999 [14]
IOSE-C9, IOSE-C10, IOSE-C21	OSE	I	Retroviral transfer of hTERT	CK-7, CK-8, CK-14, CK-16, CK-18 CK-19, CA125, E-cadherin	Li et al., 2007 [15]
NOSE4, NOSE11, NOSE19L3	OSE	P	–	AE1/AE3, CA125, CK-7, factor VIII EpCAM, E-cadherin, FSP	Lawrenson et al., 2009 [16]
OE-E6/E7	FT	I	Retroviral transfer of E6/E7 HPV16	CK-19	Lee et al., 2001 [17]

Source: FT — fallopian tube epithelium, OSE — ovarian surface epithelium; *type*: I — immortalised cell line, P — primary cell line

available WIT-T (Cellaria) medium, dedicated to human breast epithelial cell culture, contains the addition of serum and epidermal growth factor (EGF), insulin, hydrocortisone, cholera toxin, and for cells derived from endometrioid or mucinous cancer also 17-β-oestradiol (variant OCMIe). According to Ince et al., the OCMI medium allows stable lines of ovarian cancer to be obtained in 95% of cases [20].

Stable cell lines

Established cell lines are the most commonly used models in cancer research. Their application has contributed to significant progress in the understanding of cancer biology. Cell line can be regarded as stable when it has been passaged *in vitro* at least 60 times, has a stable genetic profile, proliferates well, is viable, and can be kept in culture without problems. In databases such as The Cancer Cell Line Encyclopedia (CCLE) or The Cancer Genome Atlas (TCGA), over 1000 different tumour cell lines, including several dozen ovarian cancer lines, have been catalogued so far.

Stable tumour cell lines fairly accurately reflect the spectrum of genetic changes in primary tumours. However, the immortalisation process and long-term *in vitro* culturing can affect their molecular profile. Cell lines made available today by professional repositories have a defined genetic profile; the most frequently used for this purpose is the analysis of the length of selected repetitive DNA sequences (short tandem repeats, STR). Research laboratories should verify the STR profile of their cell lines every few years and in case of non-compliance should acquire a new cell tranche from an authorised repository. More and more often, the editors of scientific journals require the source of cells to be stated and do not accept the use of cells with an unverified genetic profile.

Many commonly used cell lines were established several decades ago. In some cases, only modern molecular analyses have detected mistakes in their classification. Probably some lines were incorrectly classified initially or they were exchanged with others. Such situations were even detected in the NCI-60 panel, comprising 60 cell lines established in the National Cancer Institute at Bethesda, commonly used for preclinical studies of new drugs [21]. An example is the MDA-MB-435 line, considered for many years as a breast cancer line. Based on the evaluation of the gene

expression profile [22], karyotype analysis, comparative genomic hybridisation (CGH), and single nucleotide polymorphisms (SNP) analysis [23], this line was found identical to the M14 melanoma cell line. The discussion about the origin of both these lines is still ongoing [24]. Another breast cancer line, known as MDA-N, was found, based on molecular analysis, to be identical to the MDA-MB-435 line. In turn, the MCF-7/ADR-RES line, described as an Adriamycin (doxorubicin)-resistant variant of the MCF-7 breast cancer line, is probably a variant of the ovarian cancer line OVCAR8 [25]. It also turned out that many classic cell lines are contaminated with admixture of HeLa cervical cancer cells — the first-ever stabilised cancer cell line [26, 27]. The best option while designing the experiments is to opt out of such uncertain cell models.

Cellular models for ovarian cancer research

In the case of ovarian cancer research, it is particularly important to precisely specify the origin of cell lines, because individual histological types in fact represent distinct disease entities. Unfortunately, awareness of this phenomenon is not universal. Moreover, many of the commonly used ovarian cancer cell lines have an unclear histological origin — either indefinite from the beginning or challenged today during in-depth analyses.

In 2013, a study by Anglesio et al. was published, in which attention was drawn to the need to re-classify available ovarian cancer cell models in terms of their histological origin [28]. Another two publications, by Domcke et al. [29] and Beaufort et al. [30], aimed at organising knowledge about available cellular models and sensitising researchers to the problem of their histological origin, by analysing a panel of dozens of ovarian cancer cell lines. Despite the exhaustive molecular, morphological and genetic analyses, the origins of many lines have still not been precisely determined.

As described in part entitled "heterogeneity of ovarian cancer", the most common type of ovarian cancer is high-grade serous cancer (HGSOC), which also has the worst prognosis. Therefore, it should be expected that this type of histology is the main subject of basic and preclinical research. Analysis of publications indexed in the PubMed database indicates that SKOV3, A2780, OVCAR3, CAOV3, and IGROV1 lines are the most frequently quoted ones; however, among them, there is no good HGSOC model [29].

The SKOV3 line is usually considered as a model of serous cancer. However, in the original publication, presenting its derivation, it has only been briefly described as "an adenocarcinoma cell line derived from an ascitic fluid of ovarian cancer patient" [31]. In turn, the A2780 line was described as being derived from endometrioid adenocarcinoma [32], and further studies confirm this classification.

Domcke et al. used the data from public repositories — CCLE and TCGA — to compare gene expression profiles in cell lines and postoperative ovarian cancer samples. Additionally, taking into account the genetic profiles of cells (presence of specific mutations and variability of the copy number of DNA — copy number variation, CNV), the authors proposed a ranking of 47 cell lines in terms of their suitability as HGSOC models. SKOV3 and A2780 cell lines received the "unlikely HGSOC" label (do not correspond to HGSOC) [29]. The cells of these lines do not have the main features of HGSOC, such as high levels of CNV and the presence of mutations in *TP53* and *BRCA1* or *BRCA2* genes. Instead, they have mutations in non-HGSOC genes, such as *ARID1A* (characteristic for clear-cell and endometrioid cancer) and *PIK3CA* (associated with clear-cell cancer).

Anglesio et al. also explicitly question the suitability of the SKOV3 and A2780 lines as HGSOC models [28]. Beaufort et al. classify the SKOV3 and A2780 lines as derived from clear-cell or endometrioid cancer ("putative histology: endometrioid/clear-cell") [30]. Shaw et al. have shown that tumours that develop from SKOV3 cells after administration to nude mice have a clear cell morphology with accumulation of glycogen in the cytoplasm (microscopic image of "light" cells) [33]. A similar image was observed in three-dimensional culture (3D) of SKOV3 cells [34].

In conclusion, recent studies have confirmed that A2780 cells are derived from endometrioid cancer and have shown that SKOV3 cells are most likely to represent clear-cell cancer. The classification of SKOV3 cells is still not completely unambiguous, as some authors report the presence of *TP53* mutation, which is a typical feature of serous cancer.

OVCAR3 is the third most cited ovarian cancer line. It was obtained from ascitic fluid from a patient with recurrent ovarian cancer diagnosed as "poorly differentiated papillary adenocarcinoma" [32]. Both OVCAR3 and CAOV3 have the *TP53* gene mutation; however, according to Domcke et al., in terms of other features, they differ from the HGSOC characteristics [29]. Other researchers recognise that OVCAR3 cells are likely to represent HGSOC [30, 34].

The IGROV1 line, the last of the five most frequently cited, shows a hypermutator phenotype, and in hierarchical clustering, based on the gene expression profile, it is located away from ovarian lines, and close to cell lines originating from lung, liver, stomach, and small intestine tumours [29]. In the analysis of Beaufort et al. IGROV1 cells were assigned with the label "mixed histology" [30]. So, it is a line that is difficult to classify, and therefore it is better to give up on it when designing the research, in favour of other, more reliable models.

OAW42 and ES2 cell lines are less frequently used in ovarian cancer research, and their histological origin is

also unclear. The ES2 line is sold by the ATCC (American Type Culture Collection) as a model of clear-cell cancer; however, the histology of the primary tumour is not described in the source article [35]. Based on molecular features, Beaufort et al. concluded that ES2 cells correspond to clear-cell carcinoma [30], whereas Angelsio et al. question this histological type based on *in vivo* studies: in ES2 cell tumours they did not observe cells with a light, glycogen-rich cytoplasm [28]. In turn, Domcke et al. classify ES2 cells as "possibly HGSOC" [29].

The OAW42 line is described in the source publication as derived from serous ovarian cancer. Modern studies mostly confirm the serous type [30, 36] but not the high-grade type. In a publication by Domcke et al. this line received the label "unlikely HGSOC" [29]. Lee et al. assessed that the architecture of structures created by OAW42 cells in 3D culture corresponds to well-differentiated (G1) serous cancer [34]. However, the presence of mutations in *ARID1A* and *PIK3CA* genes (characteristic of endometrioid and clear-cell cancers) raises some doubts [29, 30]. So, this is another uncertain model of ovarian cancer, which should be replaced with recently introduced cell lines of certain origin.

As shown above, there is no reliable HGSOC model among the most commonly used ovarian cancer lines: two lines are derived from serous cancer, but not necessarily of high-grade type (OVCAR3 and CAOV3), the SKOV3 line is probably derived from clear-cell cancer and the A2780 line originates from endometrioid cancer; IGROV1 may be derived from another organ, two models have an uncertain histological origin (ES2 and OAW42).

It would be reasonable to give up the use of lines of unclear origin, but paradoxically they are still widely used. There may be several reasons for this phenomenon. Some researchers are probably not aware of the problem. Sometimes technical aspects dictate the popularity of a given line — high cell viability, low culture requirements, fast divisions, etc. A certain argument for using these lines is also the fact that they are well characterised and have extensive literature documentation to which research results could be related.

Instability of cellular models

Another problem may be the instability of cell lines in *in vitro* culture. Many lines have been in use since the 1970s and 1980s. In various laboratories around the world, and even in different repositories — under one name — there are different clones of the same cell line.

Beaufort et al. compared two variants of SKOV3 and A2780 cells — from the European Collection of Authenticated Cell Lines (ECACC) repository and from the academic laboratory where they have been propagated for years. Most analyses yielded the same results for both variants, but differences were also observed, e.g. regarding sensitivity to docetaxel and paclitaxel in the case of the A2780 line, and sensitivity to paclitaxel, carboplatin, doxorubicin, and gemcitabine in the case of SKOV3. Two variants of SKOV3 also differed regarding the presence of mutations in the *HRAS* and *APC* genes, and the level of EpCAM protein expression. In contrast, the A2780 variants differed in the *BRCA2* gene mutation.

Another example of such differences is *TP53* gene mutation in the SKOV3 line. In the publication by Beaufort et al. two methods of mutation detection were used: deep sequencing of selected amplicons and exon sequencing using the Supported Oligo Ligation Detection (SOLiD) method. In the SKOV3 line the frameshift mutation was detected only by the deep sequencing method (c.del267C) [30]. The presence of this mutation in SKOV3 cells has been previously described by the Ikediobi team [37]. In our studies we detected this mutation in SKOV3 cells from the American collection (ATCC) using Sanger's sequencing [19]. In a publication by Elias et al. [38] the presence of an (unspecified) mutation/deletion of the frameshift in a cisplatin-resistant variant of SKOV3-cis line was mentioned. In turn, Anglesio et al. did not observe *TP53* mutation in SKOV3 cells [28], which was referred to in the publication by Ince et al. [20]. Domcke et al. referred to data from the CCLE encyclopedia, which also does not record *TP53* mutation in the SKOV3 line [29].

The described discrepancies may be the result of many years of culturing; the SKOV3 line has been in use since 1973, and currently there are many different clones around the world. Another reason may be the use of different mutation detection methods by different authors. In addition, many authors refer to the results of other authors' research and do not verify them experimentally.

Another example of differences in established cell models is WT1 marker expression. Ince et al. did not observe the expression of WT1 in the A2780 line [20], while in our experiment we detected single WT1-positive cells. The differences also concern the expression of EpCAM in the OVCAR3 line — Domcke et al. obtained a negative result [29], while we observed a moderate reaction in all cells [19]. The results of our detection of CD44 marker expression were also different in comparison with the results of Beaufort et al. [30] for three cell lines: SKOV3, OVCAR3, and OAW42 [19].

It is worth noting, however, that three types of cell morphology (epithelial, spindle, and round type) described by Beaufort et al. [30] are probably a fairly stable feature and characteristic of various cell lines. In our studies we had identical observations: SKOV3, OAW42, and OVCAR3 cells showed epithelial morphology, ES2 had the shape described as spindle, and

A2780 — round. Beaufort et al. showed a significant correlation between cell morphology and the origin of the cell line — 14 out of 19 lines with epithelial morphology were from ascitic fluid. In addition, cells with epithelial morphology were more often derived from serous cancer (83%) compared to round (33%) and spindle cells (56%). The morphological type also correlated with the treatment with platinum derivatives — 10 out of 14 patients from whom epithelial cells were derived had previously received chemotherapy based on platinum compounds [30]. The OVPA8 line we derived shows epithelial morphology and has the characteristics attributed to this morphological type — it originated from ascitic fluid, from a patient with serous ovarian cancer, who had been previously treated with platinum derivatives [19].

Cellular models of high-grade serous ovarian cancer (HGSOC)

As can be seen from the above-mentioned data, a large portion of ovarian cancer research was performed on cell lines that do not correspond to HGSOC. This is due to two reasons: firstly, until recently, there was no knowledge about the fundamental differences between the histological types of ovarian cancer, and secondly, there is a lack of well-defined HGSOC cell models. The practical aspects are probably not without significance: cell lines that have low culture requirements, a short division cycle, good growth of tumours after inoculation *in vivo*, etc. are more willingly chosen. This may partly explain the huge popularity of the SKOV3 line.

According to Domcke et al., the best HGSOC models are the relatively unknown KURAMOCHI and OVSAHO cells [29]. These two lines and JHOS4 (also recommended by Domcke) were extensively tested by Elias et al. [38], who confirmed the features of HGSOC. It transpired, however, that the cells of these lines are characterised by certain limitations, such as poor growth in immunodeficient mice (SCID), especially in the case of JHOS4 lines [38].

As mentioned above, Ince et al. have developed an OCMI culture medium that enables the efficient establishment of ovarian cancer cell lines. Five new cell lines were derived using OCMI: OCI-P5x, OCI-U1a, OCI-P8p, OCI-P2a, and FCI-P2p, from patients with confirmed HGSOC [20]. These lines are provided by the Sylvester Comprehensive Centre Life Tumour Culture Core at the UM Miller School of Medicine in Miami. The first publications in which these lines were used are already available [39, 40]; however, there are still no publications directly devoted to HGSOC.

The OVPA8 line, recently derived by our team, is another HGSOC model that will soon be made available through the ECACC (accession no 19061601 and 19061602). This line has important practical advantages, such as relatively fast growth (doubling time — 44 hours) and resistance to unfavourable culture conditions, such as high confluence or old culture medium [19].

Models for studies on molecular background of chemotherapy resistance

Chemotherapy plays an important role in the treatment of advanced ovarian cancer. In most cases, the response to treatment is very good; primary tumour chemoresistance is a rare phenomenon. However, the resistance in relapse is a problem.

Studies on the molecular basis of the acquired chemoresistance are based on cell lines that have different sensitivity to cytotoxic drugs. Many cell lines derived from peritoneal fluid from patients with ascites are resistant to platinum compounds and other drugs. At that time, clonal selection has already took place and only cells tolerating high concentration of drugs survive; for example, cisplatin-resistant KURAMOCHI or OVPA8 cells; in the latter the IC50 for cisplatin is 16.23 μM [19].

Lines derived from primary tumours tend to be susceptible to cytotoxic compounds and can be used to obtain a cell variant that is resistant to the study drug. An example is the A2780 cell line derived from the tumour before the start of chemotherapy. The cells of this line are sensitive to cisplatin and paclitaxel. However, numerous variants have been developed that have resistance to these and other drugs (e.g. topotecan, doxorubicin, or auranofin). The IGROV1 line also comes from an untreated patient and is susceptible to cisplatin, and it has numerous drug-resistant variants created in laboratories. The SKOV3 line comes from a patient who has been treated with thiotepa, and these cells are sensitive to platinum derivatives. Numerous cell variants resistant to cisplatin, carboplatin, etoposide, paclitaxel, vinblastine, or vincristine have been derived for research purposes (Table 3).

Classical chemoresistance mechanisms include drug clearance by ABC transporters, glutathione detoxification, intensification of pro-survival signalling, efficient repair of DNA damage, and suppression of apoptosis in cancer cells. More recent studies indicate that ovarian cancer has many more complex mechanisms responsible for the development of chemoresistance. Other important factors include the presence of cancer-associated fibroblasts (CAF) [68, 69], changes in the protein composition of the extracellular matrix [70, 71], epithelial-mesenchymal transition (EMT) [72], the presence of stem cells [73], as well as epigenetic mechanisms [74–76]. Susceptibility to chemotherapy and prognosis may also be associated with a specific gene expression profile in the tumour, although the results of genomic testing are not consistent [77–79].

Table 3. Ovarian cancer cell lines used for research on drug resistance mechanisms

Primary line	Origin	Sampling time	Chemotherapy	Cellular variants	Response to cytostatics: Sensitivity	Response to cytostatics: Resistance
A2780	T	P	N		CP, P	
				A2780^{AF-R} (Landini, 2017) [41]		Aura
				A2780^{CP} (Behrens, 1987) [42]; A2780^{CIS} (Masuda, 1988) [43] ; A2780^{CR1}, A2780^{CR2} (Januchowski, 2014) [44]; A2780^{C30}, A2780^{C200}, A2780^{CP70} (Sak, 2015) [45]; A2780^{C12} (Sun, 2018) [46]		CP
				A2780^{ADR} (ECACC) [47]; A2780^{DR1}, A2780^{DR2} (Januchowski, 2014) [44]		D
				A2780^{PTX} (Han, 2013) [48]; A2780^{PR1}, A2780^{PR2} (Januchowski, 2014) [44], A2780^{TR}, A2780^{PTX10} (Sak, 2015) [45]		P
				A2780^{W1TR1}, A2780^{W1TR2} (Januchowski, 2014) [44]		Topo
COLO-704	A	R	l.d.			
				COLO-704^{rCDDP1000} (RCCLC) [49]		CP
ES2	T	P	N			
				ES2^{PR20} (Jazaeri, 2013) [50], ES2^{C12} (Sun, 2018) [46]		CP
				ES2^{TR160} (Ho, 2018) [51]		P
IGROV1	T	P	N		CP	
				IGROV1$^{Pt0.5}$, IGROV1^{Pt1} (Perego, 1996) [52], IGROV1^{CP} (Stewart, 2006) [53], IGROV1^{CDDP} (Stordal, 2012) [54]		CP
				IGROV1^{OHP} (Benedetti, 2008) [55]		OP
				IGROV1^{MX3} (Maliepaard, 1999) [56]		MK
				IGROV1^{T8} (Maliepaard, 1999) [56]		Topo
KURAMOCHI	A	R	CP			CP
OAW28 (41M)	A	R	l.d.			
				41M^{cisR} (Judson, 2012) [57]		CP
OAW42	A	R	CP			
				OAW42^{A} (Redmond, 1993) [58]		CP, D, EP, TP, WB, WK
OV90	A	l.d.	l.d.			
				OV90^{C-A}, OV90^{C-D} (Sherman-Baust, 2011) [59]		CP
				OV90^{D-6}, OV90^{D-7} (Sherman-Baust, 2011) [59]		D
				OV90^{P-3}, OV90^{P-7} (Sherman-Baust, 2011) [59]		P
OVCAR3	A	R	CF, CP, D			CP, P
				OVCAR3^{DDP} (Liu, 2017) [60]		CP
OVCAR4	A	R	CP			CP
PEO1	A	R	CP			
				PEO1^{CDDP} (Macleod, 2005) [61]		CP
SKOV3	A	R	T			
				SKOV3^{ip1} (Yu, 1993) [62]	P	
				SKOV3^{CDDP-P} (Yan, 2007) [63], SKOV3^{PR25} (Jazaeri, 2013) [50]		CP
				SKOV3^{VP} (Kubota, 1994) [64]		EP
				SKOV3^{CBP} (Li, 2004) [65]		MK
				SKOV3$^{Taxol-P}$ (Yan, 2007) [63], SKOV3^{TR} (Lee, 2015) [66]		P
				SK VCR$^{0.015}$, SK VCR$^{0.1}$, SK VCR$^{0.25}$, SK VCR$^{2.0}$ (Bradley, 1989) [67]		WK
OVPA8	A	R	CP, KP, P		P	CP

Origin: A — ascites, T — tumour; *sampling time*: P — primary disease, R — relapsed disease, l.d. — lack of data; *cytotoxic agents:* Aura — auranofin, CF — cyclophosphamide, CP — cisplatin, D — doxorubicin, EP — etoposide, KP — carboplatin, MK — mitoxantrone, OP — oxaliplatin, P — paclitaxel, T — thiotepa, Topo — topotecan, TP — tenoposide, WB — vinblastine, WK — vincristine, N — untreated

Cancer stem cells

The theory of cancer stem-like cells (CSLC) assumes the existence of a specific population of cells with the ability of self-renewal and differentiation towards all tumour cell populations. These cells have an increased clonogenic potential and the ability to form spheroids *in vitro* and potential for tumour development (tumorigenicity) *in vivo*. It is postulated that in ovarian cancer, tumour stem cells are responsible for primary tumour development, as well as intraperitoneal dissemination of the disease, its recurrence, and chemoresistance (reviewed in: [80, 81]).

Attempts to isolate ovarian cancer stem cells from a tumour or cell culture are based on the detection of specific markers, assessment of functional features, and clonogenic potential as well as tumorigenicity. Among the proposed CSLC markers are proteins typical for embryonic stem cells, such as NANOG, OCT4, NESTIN, ABCG2, or BMI1 and surface markers CD133, CD117, CD44, CD24, and EpCAM. Some authors indicate aldehyde dehydrogenase (ALDH1) activity as typical for CSLC [82]. Another functional feature attributed to CSLC is the activity of ABC transporters, which allows the removal of cytotoxic compounds and other substances. This feature is used for selection by flow cytometry (Fluorescence-Activated Cell Sorting, FACS). The cells that efficiently remove the Hoechst 33342 dye create in the FACS cytogram a so-called side population (SP). Some studies have confirmed that SP cells have greater tumorigenicity.

Unfortunately, no studies so far have allowed the identification of a reliable set of markers to isolate ovarian cancer stem cells. Many studies indicate that the phenotype of these cells can be very different. The most frequently proposed markers are CD133, CD44, CD24, and CD117 in combination with ALDH1 activity. It is accepted that CSLCs comprise a small percentage of primary tumour cells. Paradoxically, the expression of CD44 or CD24 is observed in a very large percentage of tumour cells. This may be the effect of phenotype plasticity of tumour cells that undergo epithelial-mesenchymal transition (EMT) and further changes towards the undifferentiated/stem-like phenotype [83]. Probably not all cells expressing these markers have functional features of CSLC. However, it has been shown that high expression of CD133, CD117, CD44, or CD24 may correlate with adverse clinical and pathological features (e.g. poor histological differentiation, higher clinical stage, chemoresistance, or shorter survival time).

The theory of tumour stem cells has important implications in therapy: it is postulated that CSLC can survive chemotherapy and give rise to relapse. Targeting cancerous stem cells can therefore be an attractive therapeutic option [18]. For many CSLC markers, inhibitors have already been developed, which are currently tested in preclinical studies (extensive review by Klemb et al. 2018 [81]).

Mesenchymal stem cells

In many cancers, the presence of mesenchymal stem cells (MSCs) is detected. In ovarian cancer, these cells probably come from visceral fat [84, 85].

The studies of Klopp's team have shown that stem cells of visceral adipose tissue isolated from the omentum stimulate ovarian cancer cells in *in vitro* experiments — they stimulate their proliferation, migration rate, and chemo- and radio resistance [84]. Buckahnovic's team observed that cancer-associated mesenchymal stem cells (CA-MSC) isolated from the tumour stimulate proliferation, expression of stemness markers, and increased chemoresistance of ovarian cancer cells in *vitro* [86]. In the opinion of these researchers, CA-MSC present in ovarian cancer come from the omentum. Normal MSCs migrate to the tumour and are converted to CA-MSC under the influence of the local microenvironment, including factors secreted by tumour cells and hypoxia. CA-MSCs have altered the expression pattern of over 1000 genes compared to normal MSCs from adipose tissue. Coffman showed that the origin of MSC is crucial for interactions with cancer cells. MSCs from the bone marrow stimulate the proliferation of breast cancer cells, but not ovarian cancer cells. However, ovarian cancer cells respond to MSC stimulation from visceral fat (omentum). Conversely, the breast tumour microenvironment leads to the transformation of MSCs from the bone marrow into CA-MSC but does not exert such an effect on MSC from adipose tissue. These processes may be associated with tissue-specific metastasising of breast cancer to the bone, and ovarian cancer to the peritoneum [85]. Perhaps this also explains the discrepancies in the literature; on one occasion the stimulating effect of MSC on cancer cells was observed, and at other times an inhibiting effect (reviewed by Klopp et al. [87]). Different effects could be related to the type of MSC used.

Three-dimensional cellular models (3D)

The previously discussed *in vitro* models relate to cell culture in a monolayer (two-dimensional culture, 2D), which is technically more convenient and easier, but this differs greatly from the physiological conditions in the tumour. The main limitation of this model is the lack of a typical microenvironment. This is particularly important when testing new drugs; that is why the results obtained in the 2D model are often not confirmed in further *in vivo* studies [88, 89].

A partial solution to this problem comes in the form of three-dimensional (3D) *in vitro* models. They fill the

gap between two-dimensional cell culture and animal models. On the one hand, the 3D models allow partial simulation of environmental features *in vivo*, and on the other hand, they offer the majority of the advantages of traditional cell culture.

3D models are constructed by creating the conditions for cell growth that promote the formation of so-called spheroids (3D structures) or by implanting cells into three-dimensional scaffolds, composed of extracellular matrix proteins or synthetic biomaterials [89, 90].

Lee et al. used poly(2-hydroxyethylmethacrylate) (polyHEMA) coated plates for induction of spheroid formation and obtained 3D cultures for 31 different ovarian cancer lines. Comparison of biological and molecular features of cells in 2D and 3D cultures as well as in the form of mouse xenografts showed that 3D models to a greater extent reflect the tumour characteristics. Cells in 3D culture were characterised by slower proliferation and greater chemoresistance. There were also differences in the expression of selected biomarkers: in the 3D model, higher expression of E-cadherin and β-catenin and lower expression of vimentin were observed, as compare to 2D culture. Only 30% of the tested lines expressed WT1, CA125, and PAX8, with CA125 and PAX8 having increased expression and WT1 reduced in 3D cultures [34].

Heredia-Soto et al. evaluated ovarian cancer spheroids produced by cells of 16 different lines. Spheroids with a diameter of 400 μm allowed the diffusion level of nutrients and oxygen characteristic for a tumour depth of 100 μm to be obtained. Tests on this model could be carried out for up to 14 days without producing excessive areas of necrosis. Cytotoxicity tests showed a higher tolerance of cells to platinum-based treatment in 3D than in 2D models [91].

The unique feature of ovarian cancer is metastasis in the form of so-called implants to the peritoneum and the omentum. Omentum is a fat-rich visceral fold that covers the abdominal organs. The surface of the omentum and the peritoneum is covered by a single layer of mesothelial cells, placed on the basal membrane, made of collagen type I and IV, fibronectin, vitronectin, and laminin. The tissue stroma contains fibroblasts, immune cells, endothelial cells, and extracellular matrix proteins. Ovarian cancer implants can be initiated either by single tumour cells or by spherical cellular aggregates 30–200 μm in size, which are exfoliated from primary tumour. These structures circulate in the peritoneal fluid and can adhere to the peritoneal epithelium. The exact composition and functional features of ovarian cancer spheroids in the intraperitoneal metastasising process have not yet been recognised [91].

Barbolina et al. developed a three-dimensional model of intraperitoneal metastases in which ovarian cancer cells were cultured in 3D gels made of type I collagen. Integrin signalling initiated by collagen binding has been shown to express early growth response 1 (EGR1) transcription factor, which induces the expression of metallothionein-matrix metallopeptidase (MT1-MMP), promoting proteolytic collagen degradation and invasion of the peritoneum [92, 93]. A similar model was used by Loessner et al. to study the interaction between ovarian cancer cells and the extracellular matrix and mechanisms of drug resistance. OV-MZ-6 and SKOV3 cells were introduced into synthetic polyethylene glycol hydrogels with the possibility of modulating biophysical features (such as stiffness) and biochemical parameters (integrin binding sites, protease activity level). These gels are stable for up to 28 days, allowing for longer experiments. It was shown that the proliferation of cells in the 3D environment was dependent on the proteolytic remodelling of the extracellular matrix. Ovarian cancer cells in 3D culture showed a higher survival rate after paclitaxel treatment than did cells in 2D culture [94].

Muranen et al. used a 3D model to analyse the mechanisms of acquiring resistance of ovarian and breast cancer cells to treatment with a small molecule inhibitor of the PI3K/mTOR pathway — BEZ235. Spheroids formed on the rBM matrix (reconstituted basement membrane) and without rBM participation were tested. Cells anchored in the rBM matrix were more resistant to BEZ235; cells lacking contact with rBM were more likely to undergo apoptosis. In cells attached to rBM, a higher expression of many pro-survival signalling proteins was observed, which may explain their better adaptive response and higher resistance to the inhibitor [95].

Models of 3D cultures are also used to obtain organotypic cultures, consisting of mixed cell populations (e.g. adipocytes, macrophages, endothelial cells), which better reflects tumour physiology. These models may be created using established or primary cell lines [96]. In organotypic models, it is advisable to use labelled, e.g. fluorescent, cells. This enables visualisation as well as separation and testing of pure cell populations, e.g. for changes in mRNA, miRNA, and/or specific proteins expression level [90, 96].

Very interesting observations have been made in a mixed culture of ovarian cancer cells with primary human adipocytes containing fluorescently labelled lipids. In this system, lipid transfer from adipocytes to tumour cells was observed, which led to accelerated proliferation of cancer cells, both *in vitro* and after administration to mice. The presence of adipocytes also stimulated the migration rate and invasion of cancer cells. These results suggest that the fatty acids provided by the adipocytes may be a source of energy for ovarian cancer cells [97]. In our opinion, this phenomenon may also be related to observations indicating a poorer ovarian cancer prognosis in women with obesity [98].

An attempt was made to create a 3D model that recapitulate the carcinogenesis of fallopian tube epithelial cells. Studies by Lawrenson et al. were based on a spheroid model of primary human fallopian tube secretory epithelial cells (FTSEC), isolated from the fallopian tubes immediately after surgery. Formation of spheroids was induced by FTSEC culture on polyHEMA-coated surface. The spheroids consisted of a cylindrical layer of epithelial cells surrounding the hyaline core, resembling the extracellular matrix of the fallopian tube. The spheroids persisted in culture for 30–60 days. Gene expression profile analysis revealed changes in the expression of over 1000 genes in cells grown in the 3D model, compared to classical cultures. These were mainly genes involved in the DNA replication process and cell cycle control [99]. Using a similar technique, this team received a 3D model of immortalised, transformed primary epithelial cells covering the ovary. The described models are suitable for testing the mechanisms of carcinogenesis and origin of HGSOC, as well as for screening for potential new drugs [96].

3D culture systems, although technically more complex, are gaining more and more importance as they allow to obtain conditions closer to the real ones. The results of experiments conducted simultaneously in 2D and 3D cultures confirm a much better resemblance of ovarian cancer biology in three-dimensional cultures. The limitation of the 3D model is the lack of functional vascularisation and the lack of cells mediating the adaptive immune response [88]. Other limitations include low availability of primary cells and the short lifespan of 3D cultures [96].

Conclusions

Ovarian cancer cell lines are a convenient model for *in vitro* studies. Cell culture is relatively cheap and simple, and the molecular features of the tumour cells in the culture fairly accurately reflect the initial tumour profile. Using stable cell lines, the reproducibility of the model between different laboratories and individual experiments is relatively high.

The limitations of the cell model are the lack of typical histology of tissues, the lack of functional vascularisation, and cells mediating the adaptive immune response. There are also no endocrine, paracrine, and nerve signalling and gradients of various substances found in the living organism.

A partial solution to the above problems is three-dimensional (3D) culture in the presence of extracellular matrix proteins or bio-similar polymers, and mixed cell cultures of tumour cells with other cellular components of the tumour (organotypic culture).

Unfortunately, in the case of many popular ovarian cancer cell lines, the problem is their incomplete characteristics, found in the original articles describing their establishment. Despite the great efforts made in recent years, it has not been possible to fully verify the histological origin of several commonly used cell lines.

Moreover, among the most commonly used ovarian cancer cell lines there is not a certain HGSOC model, instead there is one line that may even come from another organ (IGROV1), two models with uncertain histology (ES2 and OAW42), and two models of serous cancer, but not necessarily high-grade (OVCAR3 and CAOV3). The most frequently used SKOV3 line is probably derived from clear-cell carcinoma, the second most popular A2780 corresponds to endometrioid cancer.

As the most reliable models of high-grade serous ovarian cancer, the two almost unknown lines KURAMOCHI and OVSAHO are currently recommended, as well as the OVCAR4 line, used more often in research [29]. Ince et al. describe five cell lines derived by them (OCI-P5x, OCI-U1a, OCI-P8p, OCI-P2a, and FCI-P2p) as having the HGSOC phenotype [20]; however, these lines have been used very rarely in science, so far. Also, the OVPA8 line derived by our team comes from a patient with histologically confirmed HGSOC, and all our molecular and genetic tests confirm this phenotype. This line will soon be made available by the ECACC (accession no 19061601 and 19061602).

When choosing a model for ovarian cancer research it is necessary to pay attention to its specificity — both advantages and limitations. There is no perfect or universal model — the best possible model should be adjusted for assumed research goals.

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