

Molecular Mechanisms of Tumor Cell Stemness Modulation during Formation of Spheroids

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Abstract—Cancer stem cells (CSCs), their properties and interaction with microenvironment are of interest in modern medicine and biology. There are many studies on the emergence of CSCs and their involvement in tumor pathogenesis. The most important property inherent to CSCs is their stemness. Stemness combines ability of the cell to maintain its pluripotency, give rise to differentiated cells, and interact with environment to maintain a balance between dormancy, proliferation, and regeneration. While adult stem cells exhibit these properties by participating in tissue homeostasis, CSCs behave as their malignant equivalents. High tumor resistance to therapy, ability to differentiate, activate angiogenesis and metastasis arise precisely due to the stemness of CSCs. These cells can be used as a target for therapy of different types of cancer. Laboratory models are needed to study cancer biology and find new therapeutic strategies. A promising direction is three-dimensional tumor models or spheroids. Such models exhibit properties resembling stemness in a natural tumor. By modifying spheroids, it becomes possible to investigate the effect of therapy on CSCs, thus contributing to the development of anti-tumor drug test systems. The review examines the niche of CSCs, the possibility of their study using three-dimensional spheroids, and existing markers for assessing stemness of CSCs.

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INTRODUCTION

Cancer stem cells (CSCs) are a small subpopulation of tumor cells [1-3], which have the properties of stem cells (SCs) [4, 5]. SCs and CSCs have similarities and differences. The similarities include identical cell surface markers as well as ability to differentiate and self-renew using common signaling pathways [6]. One of the differences between SCs and CSCs is the degree of their dependence on the niche in which they are located. Unlike SCs, CSCs can change the niche with the help of signals stimulating proliferation. SCs are stable and contain a normal diploid genome, but CSCs are aneuploid with

chromosomal rearrangements. In addition, SCs tend to be dormant and have relatively long telomeres, which is not characteristic of CSCs.

Presence of CSCs in the tumor tissue determines formation of phenotypically different subclones and leads to differentiated cell composition of the tumor [7]. Difference between the tumor growth and tissue renewal with participation of stem cells lies in the further developmental pathway of the transient-amplifying cells: usually they differentiate and die, but in the case of tumor cells they accumulate in the body and accelerate tumor growth [8].

Unlike the differentiated tumor cells, which constitute a significant part of the tumor and use glycolysis

Abbreviations: ALDH, aldehyde dehydrogenase; CSCs, cancer stem cells; ECM, extracellular matrix; EGF, epidermal growth factor; EMT, epithelial-mesenchymal transition; EpCAM, epithelial cell adhesion molecule; EV, extracellular vesicles; JAK/STAT, janus kinase and transcription pathway activator; IL, interleukin; MSC, mesenchymal stem cells; PI3K/AKT/mTOR, phosphatidylinositol-3-kinase and serine-threonine protein kinase; TME, tumor microenvironment.

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Table 1. Specific CSCs markers for different types of cancers

CSC markers	Organ	References
CD133 ⁺ , CD49f ⁺ , CD90 ⁺ , epidermal growth factor receptor (EGFR) ⁺ , neuron-specific antigen A2B5 ⁺ , cell adhesion molecule L1 (L1CAM) ⁺	brain	[19-23]
CD133 ⁺ , CD24 ⁺ , aldehyde dehydrogenase (ALDH) ⁺ , CD44 ⁺ /CD117 ⁺ , epithelial cell adhesion molecule (EpCAM) ⁺	ovaries	[19, 24-26]
CD133 ⁺ , EpCAM ⁺ , CD117 ⁺ , α 2 β 1 ⁺ , ALDH ⁺ , CD44 ⁺ , histone-lysine-N-methyltransferase EZH2 ⁺ , C-X-C chemokine receptor type 4 (CXCR4) ⁺ , E-cadherin (ECAD) ⁺	prostate	[19, 27-29]
CD133 ⁺ , CD44 ⁺ , CD166 ⁺ , CD24 ⁺ , EpCAM ⁺	colon	[19, 30, 31]
CD133 ⁺ , CD44 ⁺ , CD24 ⁺ , EpCAM ⁺ , tyrosine protein kinase cMet ⁺	pancreas	[19, 32-34]
CD44 ⁺ , CD90 ⁺ , CD206 ⁺ , antigen OV-6 ⁺	liver	[19, 35-37]
CD20 ⁺ , CD271 ⁺ , ALDH ⁺ , CD133 ⁺	skin	[19, 38, 39]
CD133 ⁺ , adenosine triphosphate (ATP)-binding cassette transporter ABCG2 ^{high} , CD166 ⁺ , CD90 ⁺ , CD87 ⁺ , ALDH ⁺ , CD44 ⁺	lung	[19, 40-42]
ALDH1 ⁺ , CD24 ⁺ , CD44 ⁺ , CD90 ⁺ , CD133 ⁺ , α 6-integrin ⁺	mammary gland	[19, 43, 44]

as a way of energy production, CSCs possess a distinct metabolic phenotype, which, depending on the cancer type, can have highly glycolytic phenotype or phenotype inherent to normal cells – oxidative phosphorylation [9]. The distinctive feature of CSCs is their oncogenic activity, which allows them to form tumors when transplanted to laboratory animals, which is impossible in the case of transplantation of normal SCs [10].

CSCs can influence metabolism of the neighboring cells, supply nutrients and create favorable conditions for tumor growth. Presence of CSCs in the tumor microenvironment (TME) leads to formation of the heterogeneous cell populations with high plasticity potential [11, 12] and high resistance to stress factors in TME (such as low oxygen or nutrient levels) [13, 14].

Proportion of CSCs in the tumor tissues is very small and usually does not exceed 2% of the total tumor mass. CSCs are characterized by certain markers depending on the type of tissue in which they are located (Table 1). About 73% of the surface markers of CSCs are present on the membranes of embryonic or adult stem cells and are rarely expressed on the membranes of differentiated cells [15]. The leukemia stem cells have been shown to possess the CD34⁺CD38⁻ phenotype, in which absence of CD38 distinguishes these cells from the normal hematopoietic stem cells (HSCs) [16]. Singh et al. showed the presence of CD133⁺ HSCs in the glioblastoma tumors [17], which are resistant to the chemotherapeutic agent temozolomide compared to the CD133⁻ cells [18].

In addition, not only surface antigens but also microRNAs (miR-21, miR-210, miR-34a, miR-16, etc.) can be CSCs markers [45].

Stemness refers to the general molecular processes underlying properties of the SC self-renewal and generation of differentiated progeny. Stemness allows maintaining cell population and balance between dormancy, proliferation, and regeneration [46]. It is known that the tumor cell stemness is manifested by the heredity maintenance, ability to survive stress and chemotherapeutic drugs influence, and is a key feature of cancer progression [47, 48]. It was shown that the CD133⁺ stem cells of colon carcinoma are resistant to fluorouracil and oxaliplatin in contrast to the CD133⁻ cells [49].

Understanding genetic and epigenetic changes in tumor cells that are transformed into stem cells is the key to developing new therapeutic approaches. By expanding the knowledge about pathophysiology of CSCs in tumor it would be possible to create qualitative model systems that provide applied knowledge about molecular properties of the malignant tumors [46]. Xenografts are commonly considered as a model for studying CSCs stability. For example, the xenografts obtained from pancreatic tumor tissue of patients treated with gemcitabine showed resistance to the therapeutic agent due to CSCs [50]. In the T-cell acute lymphoblastic leukemia, xenografts had a stronger genetic similarity to the recurrence samples than to the main tumor at the time of diagnosis, which may imply natural selection of resistant clones during xenografting [51].

However, there are still many questions remaining regarding CSCs. One of the biggest challenges is identifying CSCs. Another challenge is understanding relation between the stemness and tumor progression. Furthermore, it is unclear how stemness contributes to the emergence of metastases and resistance to therapy [52].

Despite these challenges, progress is being made in the study of tumor stemness. Novel technologies and procedures are emerging for the detection and separation of CSCs, and scientists are commencing to elucidate molecular pathways that govern stemness in both normal and malignant cells. As this knowledge base matures, it would probably lead to better therapies and improved treatment results for cancer patients.

TRANSCRIPTION FACTORS AND STEM SIGNALING PATHWAYS

Disruption of the regulation of expression and activity of the tumor transcription factors allows the cancer cell to acquire stemness manifested by the ability to self-renew, differentiate, and form tumors *in vivo*. Such cancer stem cells have a significant potential for metastasis and resistance to therapy, which requires deep understanding of the molecular mechanisms of changes in the transcription factors associated with stemness. The main transcription factors that play a key role in the regulation of CSCs growth include OCT4, SOX2, NANOG, KLF4, and MYC [53].

OCT4 controls self-renewal and maintains pluripotency of stem cells [54]. *OCT4* overexpression has been described in some types of cancers and promotes SC self-renewal and development of drug resistance [55, 56]. *OCT4* knockdown reduces tumor cell stemness [57]. Correlation between the *OCT4* expression and colorectal cancer metastasis to liver has been established. For example, Fujino et al. investigated clinical samples and the OCT4⁺ cells of colorectal adenocarcinoma. The level of *OCT4* expression correlated with the frequency of liver metastasis in the patients, and OCT4⁺ cells had the ability to self-renew and differentiate similarly to CSCs [58]. Expression of *OCT4* by the lung cancer cells leads to polarization of M2 macrophages, inducing secretion of the macrophage colony-stimulating factor (M-CSF), which promotes tumor growth and metastasis [59].

SOX2 plays a role in the development and maintenance of undifferentiated embryonic stem cells (ESCs), performs key functions in the regulation of proliferation, self-renewal, and other processes in stem cells, and is also involved in the development of tumor cell stemness [60, 61]. *SOX2* overexpression has been established in the triple-negative breast cancer. Suppression of the SOX2 expression led to decrease in proliferation of the triple-negative breast cancer cells, repression of cell invasion ability, induction of apoptosis *in vitro*, and reduc-

tion of tumor growth and metastasis *in vivo* [62]. In the mouse xenograft model with osteosarcoma, the osteoblast-specific conditional knockout of SOX2 has been shown to cause a dramatic decrease in the incidence of tumors. All newly emerged rare tumors found in the SOX2 knockout experimental animals were SOX2-positive, indicating that they originated from the cells that avoided SOX2 deletion [63]. Also, it was discovered that the simultaneous occurrence of CD133 and SOX2 expressed together correlated with the more negative prognosis for the patient survival [64].

NANOG is a key factor for pluripotency and self-renewal of ESCs [65] and is often overexpressed in many types of cancers [66, 67]. In oral squamous cell carcinoma with increasing degree of dysplasia, NANOG expression was found to be increased, which could be an early predictor of the risk of malignant neoplasm development [68]. It was shown that the *NANOG* expression is associated with the increased aldehyde dehydrogenase (ALDH) activity and cellular radioresistance (through activation of NOTCH1 and AKT signaling pathways), as well as with stimulation of the DNA double-strand break repair [69]. Mutations in the *SPOP* gene have been shown to stop the SPOP-mediated degradation of NANOG, thereby causing increased synthesis of the stem cell markers by the prostate tumor cells and reducing patient survival [70].

KLF4 plays an important role in the regulation of proliferation, differentiation, apoptosis, and reprogramming of somatic cells [71]. KLF4 triggers expression of the telomerase reverse transcriptase (TERT) and enhances capacity of ESCs to maintain their pluripotency and continuous self-regeneration [72]. KLF4 can play a dual role in carcinogenesis, either stimulating this process or functioning as an oncosuppressor. Overexpression of KLF4 in different types of human melanomas was established. Inhibiting apoptosis through ectopic expression of KLF4 promotes higher division rate of the melanoma cells. In contrast, the KLF4 knockdown decreases melanoma cell proliferation and causes cell death. Moreover, KLF4 depletion reduces growth rate of the melanoma xenograft *in vivo* [73]. KLF4 plays an important role in regulation of the osteosarcoma cell stemness, and the KLF4-p38 MAPK signaling pathway could be a potential therapeutic target for osteosarcoma treatment [74]. Inhibition of the KLF4 expression has been demonstrated to enhance migratory and invasive properties of the non-small cell lung carcinoma. When the KLF4 expression was restored, decrease in the tumor cell invasion was noted [75]. Moreover, sensitivity of the ovarian cancer cells that were genetically modified and transduced with the lentivirus for KLF4 overexpression to the effects of paclitaxel and cisplatin was enhanced as a result of increased cell responsiveness [76].

MYC proteins (c-Myc, L-Myc, S-Myc and N-Myc) are a family of transcription factors that regulate growth,

Table 2. Influence of signaling pathways on cancer development

Name of signaling pathway	Function	Activation in tumor cells	References
Wnt/ β -catenin	regulates embryogenesis and cell differentiation	mammary gland, colon, esophagus	[84-90]
Notch	one of the most important signaling pathways for stem cell maintenance and function	breast, stomach, pancreas, colorectal, as well as hematologic malignancies	[91-93]
Hedgehog	participates in embryonic development, formation of various body systems, including nervous system, as well as organs such as lungs, heart, and intestines	lung, breast, skin, brain, liver, gallbladder, pancreas, stomach, colon, prostate, and hematologic malignancies	[94-99]
NF- κ B	participates in the cell survival, proliferation and differentiation	gastrointestinal tract, urogenital system, head and neck cancer, breast tumors	[100-102]
JAK/STAT	promotes survival, self-renewal, hematopoiesis and neurogenesis of ESCs	lungs, colon, liver	[103-109]
PI3K/AKT/mTOR	important for the cell proliferation and survival	breast, lungs, colon, bone marrow	[110-115]

Table 3. Proteins and enzymes involved in maintenance of the CSC stemness

Title	Role	References
Galectin-3	plays a role in the cell proliferation, dynamic cell transformation, migration and invasion, survival and apoptosis	[116]
Cullin 4B (CUL4B)	stimulates development and metastasis of colorectal cancer	[117]
Lysindemethylase 3A (KDM3A)	is a critical regulator of ovarian cancer stemness and cisplatin resistance	[118]
Farnesyl diphosphate synthase (FDPS)	plays an important role in maintaining glioblastoma stemness	[119]

differentiation, self-renewal, metabolism, and cell cycle of stem cells [77]. Overexpression of *MYC* is often observed in the human tumors compared to normal tissue. *MYC* dysregulation plays an important role in maintaining the number of invasive CSCs. It was found that *MYC* overexpression in the glioblastoma CSCs induces tumor cell proliferation and invasion, and inhibits apoptosis [78]. It was shown that dysregulation of *MYC*, including increase of this gene expression, leads to the development of invasive ductal carcinoma [79]. Notably, *c-MYC* activates *SOX4* expression through binding to its promoter, thus, *c-MYC* overexpression may contribute to the prostate cancer progression through the *SOX4* activation [80].

There is increasing evidence that the overactive or abnormal signaling pathways may contribute to the sur-

vival of CSCs. It was found that the malignant neoplasms are characterized by dysregulation of Wnt, NF- κ B, Notch, Hedgehog, JAK/STAT, and PI3K/AKT/mTOR signaling pathways, which play an important role in the CSCs stemness maintenance (Table 2) [53]. Moreover, the proteins of the above signaling pathways are involved in migration, development/maintenance of resistance and tumor formation, as well as in the epithelial-mesenchymal (EMT) and mesenchymal-epithelial (MET) transitions [81]. Genes of different signaling pathways can be expressed at different stages of carcinogenesis in different tumor types as well as have cross interaction in the CSCs regulation [82]. Thus, signaling pathways regulating CSCs proliferation are complex networks of signaling mediators interacting with each other [83].

Table 4. Clinical trials of new antitumor cancer therapy strategies targeting cancer stem cells

Type of cancer	Description	ID ClinicalTrials.gov
Glioblastoma	an open randomized phase II/III trial of dendritic cell immunotherapy against CSCs in the patients with glioblastoma receiving standard therapy	NCT03548571
Adenocarcinoma of lung, liver, pancreas	a randomized trial whose main objective is to determine safety of immunization with the CSCs vaccine	NCT02084823 NCT02089919 NCT02074046
Epithelial cancer of the ovaries, fallopian tube, or abdomen	a randomized controlled clinical trial to determine combination of the drugs using the ChemoID assay (the test measures cytotoxic effect of the drugs and identifies the most effective drug that can affect CSCs)	NCT03632798
Breast cancer	a randomized phase II study designed to evaluate activity of bevacizumab in combination with the conventional chemotherapy against CSCs	NCT01190345
Carcinomas, sarcomas (lung, esophagus, thymus), or germ cell neoplasms with pleuropulmonary metastases	a non-randomized phase I/II study that examines effects of the drug (CSC signal transduction inhibitor)	NCT02859415
Recurrent glioblastoma	phase I trials designed to study effectiveness of the activated autologous T cells against the CSCs antigens	NCT05341947

Also, there is quite a large number of additional molecular agents affecting the CSC stemness (Table 3).

Thus, the study of transcription factors and signaling pathways not only provides scientific knowledge in the field of stem cell biology, but also makes a significant contribution to the application of this knowledge to the creation of new therapeutic strategies (Table 4). A large number of studies are aimed at identifying targeted therapies using components of signaling pathways as targets. For example, understanding of the tumor stem-driven interactions between the breast cancer subtypes is fundamental to identifying possible therapeutic approaches. In the study by Mei et al. difference between the cell subtypes was determined using the CRISPR/dCas9 system targeting genes *OCT4*, *KLF*, *MYC*, *SOX2*. It has been shown that the cells synthesizing epidermal growth factor receptor 2 (HER2) are precursors of the luminal A cells, hence, similar antitumor drugs can be used for the therapy targeting these tumor cells [120].

STEMNESS AND TUMOR MICROENVIRONMENT

In the absence of specific microenvironmental factors, stem cells are unable to sustain their viability beyond their respective niches [121]. It is known that extrinsic stimuli can promote emergence of the new stem cells, as the cells in general retain the ability to dedifferentiate [122]. Exposure to extracellular microenvironmental

factors such as mesenchymal stem cells (MSCs), immune cells, tumor-associated fibroblasts (TAFs), extracellular matrix (ECM), vesicles, and hypoxia promotes acquisition of stemness by the tumor cell or reprogramming into CSCs [53]. Vascular endothelial growth factor (VEGF) contributes to acquisition of the tumor stemness phenotype by the cells in squamous cell skin cancer [123]. In the head and neck squamous cell carcinoma, epidermal growth factor (EGF) secreted by endothelial cells induces EMF with the cells acquiring stem cell characteristics [124]. Transforming growth factor (TGF)- β secreted by TAFs promotes ECM and increases proliferation and emergence of metastases of breast cancer cells [125].

Hypoxia also affects stemness and other properties of tumor cells by increasing synthesis of the hypoxia-inducible factor (HIF)-1 α [126, 127].

The effect of ECM proteins on stemness modulation is interesting. For example, Zhang et al. studied a fibrin gel that was used to create a three-dimensional (3D) ECM of colorectal cancer cells. The soft fibrin gel (90 Pascal) was the most effective for enrichment of tumor colonies. In particular, these tumor colonies exhibited carcinogenicity, activated the stem cell markers, and exhibited anti-chemotherapeutic properties. These structures were called tumor repopulating cells (RTCs). It should be noted that NANOG factor was induced in the 3D-cultured colon RTCs [128]. It is also known that the collagen type I (Col-I) induces tumor stemness. The work of Valadão et al. determined dependence of this

effect on the Col-I density. The results showed that high Col-I density increased the number of the CD44⁺CD24 breast cancer stem cells, but did not promote cell self-renewal or clonogenicity. Thus, it was concluded that high Col-I density by itself is not sufficient for full development of the breast cancer stem cell phenotype [129].

Factors associated with inflammation can activate the reprogramming network leading to generation of CSCs. The serum IL-6 levels were found to be higher in the patients with osteosarcoma than in the controls. IL-6 also increased proliferation and spherulation of the osteosarcoma cells as well as their invasive and migratory potential, thus contributing to formation of the cells with an EMT-like phenotype and increased chemoresistance. In a xenograft model of osteosarcoma, it was determined that the tumor size and weight were higher in the mice treated with recombinant IL-6 than in the controls [130]. It was found that IL-1 β can induce spherulation of the colorectal cancer cells that were characterized by activation of the stemness factor genes (*BMI1* and *NESTIN*) and increased drug resistance, which is a hallmark of CSCs [131]. Hong et al. showed that the tumor necrosis factor (TNF)- α promotes oral carcinogenesis associated with human papilloma virus by increasing tumor stemness [132].

Extracellular vesicles (EVs) of tumor and stromal cells mediate intercellular communication in the tumor microenvironment (TME) and participate in various stages of carcinogenesis such as proliferation, angiogenesis, metastasis, and resistance to therapeutic drugs. Because EVs carry various substances in them, they can participate in intercellular communication and play a role in the acquisition of stemness by cells. For example, EVs produced by the glioblastoma stem cells promote endothelial tube formation [133]. Similarly, in the colorectal adenocarcinoma, exosomes of TAFs promoted increase of the CSCs percentage in the tumor by activating the Wnt signaling pathway [134]. EVs from MSCs can have two effects: they can enhance tumor growth and metastasis, or they can promote cell apoptosis and cause tumor regression [135, 136].

Thus, extracellular factors released by the tumor microenvironment affect cell stemness and heterogeneity through regulation of the signaling pathways.

SPHEROIDS AS A MODEL SYSTEM FOR STUDYING CANCER STEM CELLS

To date, the use of 3D models in tumor research attracts attention of investigators. One of such models is the tumor spheroid, which can be divided into several types: (i) a multicellular spheroid first described in the early 1970s and generated by subjecting tumor cell lines to non-adherent culture dishes; (ii) oncosphere – a prototype for expansion of CSCs utilizing serum-free

medium in combination with added growth factors; (iii) an organotypic multicellular spheroid created by dissociating tumor tissue via mechanical and enzymatic methods [137].

Multicellular spheroids are used as an *in vitro* model system for studying micrometastases and avascular tumor regions. In addition, this type of spheroids is suitable for studying cell adhesion-dependent resistance.

The terms “sphere”, “tumor sphere” [138], and “oncosphere” [139] are used to denote spherical structures originating from CSCs. Remarkably, the names of spheres can be derived from the type of tissue from which they are derived. For example, spheres obtained from stem cell cultures of the brain and mammary gland have been named “neurospheres” and “mammospheres”, respectively. Studies aiming *in vitro* evaluation and characterization of CSCs are usually performed using this model [140–142].

Organotypic multicellular spheroids are engineered to mimic TME exactly, which includes tumor cells, non-tumor cells, and stromal components [140].

In the literature, these models are placed between the 2D and xenograft models, suggesting that the 3D models can fill the gaps that exist between them [140]. 3D models are characterized by high translatability of biological characteristics of the original tumors, which gives advantages when using them [143]. 3D models have architecture and metabolic responses similar to the native tumors. Histomorphological analysis shows that the 3D model, in particular a tumor spheroid, is divided into several zones, just like a natural tumor. These include the outer zone characterized predominantly by proliferating cells, the middle zone with dormant cells, and the central necrotic zone [144]. This can be explained by insufficient oxygenation, lack of nutrients, and accumulation of metabolites. The outer zone of spheroid is characterized by the large amount of lactate and minimal amount of adenosine triphosphate (ATP), mainly due to the significant consumption by the proliferating cell layer. Glucose distribution occurs uniformly in all areas of the spheroid [145].

The listed tumor spheroid types can contain different cells supporting genetic heterogeneity and interaction between the tumor cells and stroma. 3D models offer a possibility to study intercellular contacts, microenvironment, and interaction between the immune and tumor cells [146]. Experiments with the 3D cell culture could provide more accurate data on the effects of therapeutic agents, metabolic profiling, stem cell research [147], and the processes such as EMT and MET, which are involved in the regulation of stemness. Activation of several transcription factors, including SNAI1 and SNAI2, at the molecular level accounts for the effect of EMT. This effect is associated with the loss of intercellular contact molecules, such as E-CAD, as well as with acquisition of mesenchymal markers, including vimentin.

EMT has been particularly studied in various types of tumor spheroids as well as in normal stem cells, including cardiospheres, neurospheres, and retinal spheres. Additionally, EMT activation studies have been performed with the embryonic stem cells (blastulation), embryoid cells, and cells subjected to induced reprogramming [148].

Tumor spheroids can be obtained by aggregation or spontaneous self-assembly. Aggregation can be induced by forcing cell-to-cell contact using various methods, such as hanging drop, cell suspension, microwell (96-well round-bottom plates), or microhydrodynamics (i.e., encapsulation in a gel) [140].

Studies of the spheroid model systems using novel specific agents targeting Notch (MK-0752, R4733, and TR-4) [113], PI3K (CAL-101 and XL-147) [114], AKT (perifosine and arhexin) [149], and Hedgehog (BMS-863923 and IPI-926) signaling pathways are of great interest for the development of anti-tumor therapy [150]. It was found that salinomycin (ionophore polyether antibiotic) is able to selectively kill the mammary CSCs and induce epithelial differentiation of tumor cells contributing to tumor elimination [138]. Namiki et al. showed that epigallocatechin gallate, one of the most important organic substances of green tea, reduces stemness and oncogenicity of the human lung cancer cells by inhibiting AXL receptors [151].

A significant obstacle to the development of rational cancer therapies is mismatch between the model systems and the patient's body. To solve this problem, Fendler et al. used CSCs to create tumor spheroids, three-dimensional tumor organoids, and xenografts of the light cell renal cell cancer. Treatment with the Wnt and Notch signaling pathway inhibitors blocked proliferation and self-renewal of CSCs in the spheroids and organoids and inhibited tumor growth in the patient-derived xenografts in mice [152].

Thus, use of these model systems expands the possibilities for creating a screening platform for drug testing. In addition, use of 3D models opens up prospects for the development of personalized medicine. And the study of CSCs reveals their molecular mechanisms and contributes to the search for a therapeutic target for innovative therapies aimed at eliminating CSCs.

ENRICHMENT OF THE POPULATION OF CANCER STEM CELLS IN SPHEROIDS

One of the *in vitro* methods commonly used to identify CSCs and study their properties is the spherulation assay. The sphere-forming cells are stem cells. Spheroids are associated with the *in vitro* cancer stemness studies and are considered as one of the criteria to confirm the presence of CSCs [153]. It should be noted that in the literature both sphere and spheroid names are used for

this CSCs analysis. For example, Qureshi-Baig et al. cultured spheroids obtained from the clinical specimens of colorectal carcinoma. These spheroids were shown to phenotypically resemble a native tumor, retain several similar mutations related to colorectal cancer, and express *SOX2*, *OCT4*, and *NANOG*. After comparing the tissue and cell line spheroids, it was found that both types of spheroids showed similar self-renewal ability and similarly formed tumors in immunodeficient mice [154]. Human ovarian CSCs were also characterized, which demonstrated increased chemoresistance to cisplatin and paclitaxel and expression of the stem cell genes (*Notch-1*, *NANOG*, *NES*, *ABCG2*, and *OCT4*) [155].

There is a fairly large number of CSCs studies related to the use of spheroids. For example, combining mathematical modeling and RNA sequencing of the ovarian tumor spheroids formed by the "hanging drop" method allowed revealing genes associated with the CSCs appearance and support of chemoresistance and malignant phenotype. Emergence of the CSC populations in the spheroids has been shown [156]. Bahmad et al. described protocols and a list of necessary reagents for creation of prostatic tumor spheroids and various manipulations with them [157]. The prostate tumor spheroids have been shown to survive more than 30 passages without noticeable loss of their stemness. Spheroids proliferated more slowly with the significant decrease in activation of the PI3K/AKT signaling pathway compared to the DU145 monolayer cells [158].

Various factors can influence enrichment of the CSCs population in spheroids, which can be used in studies. For example, serum-free culture conditions have been used to enrich SCs spheroids. Herheliuk et al. showed the possibility of enriching a population of tumor cells [in this case, multicellular tumor spheroids (MTS)] with CSCs. Standardization of culturing protocols for the enriched MTS (eMTS) may provide an opportunity to use these cultures for identification of the drugs that can inhibit proliferation of CSCs. Serum-free medium with addition of the fibroblast growth factor (FGF), EGF, insulin, and hydrocortisone was used to enrich CSCs in spheroids. It was shown that the highest expression of CD133, CD44, CD24, and BMI-1 receptors was detected in the eMTS. eMTS were less sensitive to anticancer drugs (cisplatin, methotrexate, and doxorubicin) than the adherent cell cultures and MTS cultured under standard conditions in complete nutrient medium [159].

Similarly, human hepatoma cell lines PLC/PRF/5 and HepG2 were cultured under the conditions used for CSCs. The culture medium included serum-free DMEM/F12 medium supplemented with 100 IU/ml penicillin, 100 µg/ml streptomycin, 20 ng/ml recombinant human EGF, 10 ng/ml recombinant basic human FGF and B27, and N-2 supplements. The cells were then cultured in 6-well non-adhesive plates at a density

of no more than 5000 cells/well. As a result, the PLC/PRF/5 spherule-forming cells exhibited the properties of CSCs: self-renewal, extensive proliferation, drug resistance, and overexpression of OCT3/4, OV6, EpCAM (epithelial cell adhesion molecule), CD133, and CD44. Even a small number of cells were capable of forming tumors in the immunodeficient mice, and did not lose this property after repeated passaging [160]. It was also found that the changes in the components of the environment can play a role in the stem cell phenotype. Maliszewska-Olejniczak et al. showed that adding xeno-free or low-serum media to the 3D renal cell carcinoma culture increased expression of the genes encoding stemness factors, including *ECAD* (E-cadherin), *N-cadherin* (*NCAD*), *VEGF*, *SOX2*, *PAX2*, and *NESTIN* [161].

Different matrices can influence increase in the number of CSCs in spheroids. For example, culturing MCF-7 cells on the collagen matrices increased cell proliferation and generated a population of cells with CSCs properties [162]. Moreover, small leucine-rich proteoglycans of the extracellular matrix can play a role in regulation of the signaling pathways, drug resistance, and plasticity of the CSC-like cells [163]. It has been also shown that agar, which can be used to create non-adhesive conditions when generating spheroids, can play a role in the enriching spheroids with CSCs. This has been confirmed by the increased expression of CSCs markers, presence of the CD133⁺/CD44⁺, or SORE6⁺ cells, and increased capacity for self-renewal and carcinogenesis *in vivo* [164]. The same coating (agar) was used to cultivate spheroids from the oral squamous cell carcinoma cells, which showed expression of the stem cell markers and manifested chemoradiotherapy resistance in addition to the ability to initiate tumor formation and self-renewal [165]. Furthermore, additives such as methylcellulose and gellan gum were used to create a new culturing system based on the traditional system. The developed culturing system offers an attractive alternative method for *in vitro* CSC amplification with the potential for extensive use in CSC research and drug screening [166].

To create spheroids with the increased CSCs potential, a single cell cloning method can be applied. In particular, Muenzner et al. created single-cell HepG2 clones that formed 3D clusters of cells after 8 days of incubation that were individually transferred to the wells of a 12-well plate for cell culture. The clone no. 5 has been shown to exhibit more highly aggressive and invasive characteristics, most likely related to the significantly higher expression of the clinically relevant stem cell markers and more pronounced mesenchymal phenotype. In addition, higher metastatic potential was detected for the clone no. 5 cells compared to the parental HepG2 cell line *in vivo* using fluorescence imaging. Hence, the CSCs-enriched HepG2 clones certainly represent suitable model systems to study the role of CSCs during initiation,

progression, and drug resistance of hepatocellular carcinoma [167].

Interestingly, it is possible to study the effect of MSCs on the tumor by creating hybrids with gastric cancer cells using polyethylene glycol (PEG) *in vitro*. Such hybrids were characterized by the changes in EMT with downregulation of ECAD and upregulation of vimentin, NCAD, α -smooth muscle actin (α -SMA), and fibroblast activation protein (FAP). The hybrids had increased expression of the stemness transcription factors *OCT4*, *NANOG*, *SOX2*, and *LIN28*. Expression of CD44 and CD133 was higher in the hybrid cells than in the parental gastric cancer cells. Enhanced migratory properties and division of the hybrid cells were observed. In addition, heterotypic hybrids promoted gastric xenograft tumor growth *in vivo*. Probably, fusion between the MSCs and gastric cancer cells promotes formation of oncogenic hybrids with EMT and stem cell properties that can provide an opportunity to study the role of MSCs in gastric cancer [168].

Thus, main feature of the tumor-derived spheroids is their enrichment with CSCs or cells with stem cell-related characteristics. Since eradication of CSCs is likely to be of clinical importance because of their association with the malignant nature of tumor cells such as carcinogenicity or chemoresistance, the study of tumor-derived spheroids could provide invaluable clues for the fight against cancer. The tumor-derived spheroids could be useful for the detection of new CSCs markers, become a convenient model for better assessment of tumor cell response to drugs, cell proliferation, and morphology [161, 169]. In addition, increase of the spheroid stemness can contribute to the improvement of the screening platform of antitumor therapeutics, identification of new therapy targets, and study of CSCs biology.

CONCLUSION

Survival rates of the patients with malignant neoplasms have increased significantly over the past decades due to development of new antitumor therapeutic strategies, which include improved chemotherapeutic agents and targeted therapies. Nevertheless, therapy of the advanced stages of cancer is complicated with high cellular heterogeneity due to genetic mutations, tumor stroma, and presence of CSCs, angiogenicity, and tumor metastasis. In the tumor microenvironment, CSCs support tumor growth through activation of various signaling pathways thus mediating supply of nutrients and metabolites. Due to plasticity and self-renewal, CSCs form a group of resistant clones, thus contributing to the development of drug resistance and tumor metastases. Hence, CSCs are an important therapeutic target in the creation of new antitumor treatment strategies. Better understanding of the biology of CSCs and their

interaction with microenvironment will allow significant advances in the fight against tumor progression, recurrence, and resistance to therapy. A limiting factor in the development of effective therapies is structural complexity of the tumor, which is difficult to reproduce in the *in vitro* model systems. Modeling TME is particularly difficult, and therefore the use of 3D models can improve selection of drug candidates at the stage of their development. One such model is spheroids, which are of great interest because of their ability to recreate tumor pathogenesis through flexible modification. The use of spheroids to study CSCs will enhance prognostic potential of the cancer research and provide new opportunities for cancer therapy.

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